Towards Biological Control of Pistachio Dieback

Asmah Salowi

Thesis submitted for the degree of

Master in Agricultural Science

School of Agriculture, Food and Wine

Faculty of Sciences

University of Adelaide

Waite Campus

May 2010

TABLE OF CONTENTS

ABSTRACT

Xanthomonas translucens (*Xtp*) causes dieback disease of pistachio in Australia. The bacterium infects the vascular tissues of the trees, causing discolouration of the xylem, lesions on the trunk and major limbs, decline and, in some cases, death. Although hygiene and application of quaternary ammonium disinfectant to pruning wounds have been recommended to limit the spread of the disease, effective control methods are lacking. Biological control offers potential in managing this disease. The aims of this research were to assess the ability of selected bacteria to antagonise *Xtp* and to evaluate the ability of the synthetic peptide BP100 to suppress growth of *Xtp* in liquid medium.

Isolate KI of *X. translucens* (DAR75532), obtained from a commercial pistachio orchard in Kyalite (NSW) was used. The potential antagonists comprised one isolate of *Bacillus subtilis* and several bacteria isolated from pistachio wood and stored following indications of ability to inhibit *Xtp*.

Preliminary screening of the potential antagonists was conducted by means of an agar diffusion assay, in sucrose peptone agar (SPA) and nutrient agar (NA). Inhibition of growth of DAR75532 varied among bacterial isolates and with the culture medium used. Generally, the isolates produced larger inhibition zones on SPA than on NA and appeared to be bacteriostatic. When diffusible compounds were extracted from liquid cultures through centrifugation and filtration, there was no evidence of antibiotic activity. Further experiments demonstrated that two antagonists produced antibacterial metabolites in liquid medium. In contrast, culture filtrate of isolate 64161-7 grown in nutrient broth supplemented with yeast extract and glucose inhibited DAR75532, and filtrate from isolate PC397 grown in the same medium, or in nutrient broth supplemented with yeast extract and nutrient broth alone, was inhibitory. However, the antibiotic effect of PC397 was lost as the cell free culture filtrate was diluted. Competition was also identified as a possible mechanism, as DAR75532 was not recovered on SPA when mixed with isolates 64161-17, SUPP, *B. subtilis*, PC397, PC506 or PC507.

An *in vitro* assay was developed to evaluate the ability of the potential antagonists to reduce colonisation of pistachio wood by DAR75532. The pathogen, antagonists 64161L and PC397, or pathogen plus antagonists were vacuum-infiltrated into non-autoclaved excised pistachio twigs, before incubating for 10 days. The

pathogen and antagonists were recovered from the middle section of the wood, following soaking in saline and plating suspensions on SPA and NA supplemented with cephalexin, ampicillin and gentomicin (NA+ab). The antagonists were recovered and grew well on SPA but not NA+ab, indicating that they survived in pistachio wood. The pathogen was recovered on NA+ab and SPA, although indigenous wood-inhibiting bacteria also grew on SPA. Only PC397 was recovered from twigs inoculated with PC397 plus DAR75532. This suggested that PC397 inhibited colonisation of pistachio wood by the pathogen.

Peptide BP100 was obtained from the University of Girona, Spain. A turbidimetric-based system was first used to monitor the effect of BP100 on growth of DAR75532 over time. Multiplication of DAR75532 in sucrose peptone broth (SPB) was delayed or reduced in the presence of BP100. At low concentration, the peptide was bacteriostatic and DAR75532 colonies were subsequently recovered on SPA. Higher concentrations were bactericidal. To verify bactericidal activity, suspensions of DAR75532 treated with peptide were sampled over time and the colony forming units enumerated on SPA and compared with untreated controls. The minimum inhibitory (bacteriostatic) or bactericidal concentration of BP100 was influenced by the initial concentration of DAR75532 and by incubation time. Peptide at 2.5 μM was sufficient to inhibit growth of DAR75532 in SPB when the initial concentration was 10^6 CFU/ml, but a minimum of 5 μM was required to kill the cells. The mortality of DAR75532 three hours after treatment was 77.35% when 5 μM of peptide BP100 was applied.

Preliminary screening had identified isolate 64161-7 as having potential to inhibit DAR75532. The isolate was tentatively identified as *Pseudomonas tolaasii, P. fluorescens* or *P. putida* by the National Collection of Plant Pathogenic Bacteria (UK). Although this isolate survived well in excised twigs of pistachio, it did not prevent colonisation of the wood by DAR75532. However, PC397, likely to be a *Bacillus* sp., reduced colonisation by DAR75532. The other six potential antagonists remain to be tested on pistachio wood. The effect of medium composition, such as sugar content, on antibiotic production should also be investigated. In addition, peptide BP100 offers promise as a means of controlling pistachio dieback. The ability of the bacterial isolates and the peptide to reduce colonisation of DAR75532 in pistachio trees should be assessed in a natural system where the influence of other factors can be evaluated.

DECLARATION

This thesis contains no material which has been accepted for the award of any other degree in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by any other person, except where due reference is made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying.

I also give permission for the digital version of my thesis to be made available on the web, via the University‟s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Asmah Salowi

20 May 2010

ACKNOWLEDGMENTS

My sincere gratitude goes first to my supervisors, Associate Professor Dr Eileen Scott and Dr Danièle Giblot-Ducray for their invaluable supervision, advice and encouragement throughout my candidature. Special thanks to Professor Giora Kritzman for the assistance and advice rendered with the antibiotic and wood experiments.

I would also like to express my appreciation to Dr Steve Barnett, for helpful ideas; Jan Gooden and Judy Rishworth, for assisting with the spectrophotometer; Wuryatmo Sidik for assistance with statistical analysis.

Many thanks to all members of the Plant Pathology Research Group, especially Kueh Kiong Hook, Tu Anh Vu Thanh, Erminawati, Amanda Benger, Cathy Todd and Steve Coventry for their help and companionship in many ways.

I also wish to acknowledge the Sarawak State Government of Malaysia for their financial support and granting my study leave.

Last, but by no means least, special thanks to my husband, Yazid Bostamam, and my children, Nazran and Hannah for their never-ending support and patience during the entire course of my study.

CHAPTER ONE

Introduction and review of the literature

1.1 General introduction

Xanthomonas translucens (*Xtp*) is the causal organism of dieback disease of pistachio in Australia (Facelli *et al*., 2002; 2005). The organism was later established as *Xanthomonas translucens* pv. *pistaciae* pv. nov (Giblot-Ducray *et al*., 2009). The bacterium infects the vascular system of the tree and can be present at low levels without expressing symptoms (Scott *et al*., 2009), and therefore is difficult to control. The usual curative and preventive treatments for bacterial disease, which involve application of copper biocides, have proved ineffective. The application of quaternary ammonium bactericide on pruning wounds was shown to have potential to control the spread of *Xtp*, but has not been registered for this purpose on pistachio in Australia (Sedgley *et al*., 2006; Taylor *et al*., 2007). An alternative strategy, such as biological control, may offer a way of protecting pistachio trees from the disease.

Although biological control of *Xtp* in pistachio has not been reported, several bacterial strains isolated from infected trees inhibited *Xtp in vitro* (Facelli E. & Taylor C., pers. comm., 2008). These isolates warranted evaluation as biological control agents of *Xtp*.

As antagonistic ability *in vitro* does not necessarily reflect efficacy on the host plant, evaluation should be expanded to involve the potential antagonists, pathogen and host plants (Alabouvette *et al*., 2006). Isolates shown to have antagonistic potential *in vitro* therefore need to be tested for ability to suppress or eliminate the colonisation of wood by *Xtp*.

The definition of biological control can be extended to include the use of peptides or small proteins extracted from plants or microorganisms in disease control (Marcos *et al*., 2008). The potential of peptides has been well-studied in human health. In plant protection, a number of peptides exhibiting antimicrobial activity against plant pathogens has been evaluated *in vitro* (Rajasekaran *et al*., 2001; Ferre *et al*., 2006; Monroc *et al*., 2006), and some has also been tested on plant tissues (Badosa *et al*., 2007; Muñoz *et al*., 2007; Badosa *et al*., 2009). The effect of peptides on the growth of the pathogen causing dieback in pistachio remains to be assessed.

1.2 Introduction to pistachio

Pistachio (*Pistacia vera* L.*)* is a member of the family Anacardiaceae. The tree is dioecious, meaning that male and female flowers are produced on different trees, and both are essential to produce nuts. Trees begin to produce at 4-7 years of age and achieve their full bearing when they are between 10-12 years old. They tend to produce in alternate years, that is a heavy crop of nuts in one year, followed by an „off" year with greatly reduced yield. In spite of their requirement for specific climatic conditions to produce good and high yield of nuts, pistachio trees can survive in harsh conditions. This is due to their extensive root systems which allow them to „mine" the soil deeply for water and nutrients (Hendricks & Ferguson, 1995).

Pistachio is native to western Asia and Asia Minor, and was first introduced to Australia in 1935. The first commercial plantings were established in the early 1980s and nut production started in the 1990s. Most orchards can be found along the River Murray across the borders of New South Wales, Victoria and South Australia (Pistachio Growers Association Inc., 2008). Currently, there are about 35 pistachio growers in Australia, with about 550 hectares of trees planted. The current capacity is about 1,500 tonnes of nuts per year, which is about 75% of domestic consumption (Pistachio Growers Association Inc., 2008). The production of pistachio in Australia during the 2008-2009 season was about 1,500 tonnes, a small proportion of the 364,400 tonnes produced world-wide (Nut Producers Australia, pers. comm., 2009).

The most common pistachio cultivar grown in Australia is Sirora (Gilbert, 2005). This cultivar, released by CSIRO, produces attractive nuts with excellent flavour, and requires lower chilling hours for nut production (Robinson, 1998). As pistachios are very difficult to grow from cuttings, the trees are usually available as grafted or budded trees.

A number of diseases affect the growth of pistachio trees. Minor diseases affecting the leaves and fruits of pistachios, such as panicle and shoot blights, are caused by *Alternaria* spp. and *Botryosphaeria* spp. (Mila & Michailides, 2006). Verticillium wilt, a soil-borne disease, has been considered important in the pistachio industry, particularly in California (Epstein *et al*., 2003). The disease is caused by the fungus *Verticillium dahliae,* which colonises the vascular system. The impact of verticillium wilt on pistachio trees can be minimised by using resistant or tolerant

rootstocks, such as *P. integerimma* or UCB1 (*P. atlantica* x *P. integerrima* hybrid) (Teviotdale, 1995) and, as these are increasingly being used in Australia, the disease is not considered as a serious threat to the Australian pistachio industry.

Bacterial dieback, recently reported in Australia, is a serious disease that affects the vascular system of the tree. Some affected orchards have more than 70% of the trees showing the disease symptoms and, in one area, the disease caused death of more than 10% of the trees (Edwards & Taylor, 1998). The pistachio industry in Australia is still young, and this disease is considered an impediment to the expansion of the industry (Scott *et al*., 2009).

1.3 Introduction to the dieback disease

Pistachio dieback is a bacterial disease. It is a relatively new disease affecting the pistachio industry, which has been reported only in Australia. It was first observed in 1992 and it started causing economic losses at the onset of commercial pistachio production in 1996 (Facelli *et al*., 2002). Symptoms associated with dieback are decline, discolouration of xylem tissue in shoots of 2 or more years old, lesions on trunks and major limbs, and excessive oozing of resin from the trunk (Facelli *et al*., 2002). Growth of the tree is stunted and dieback may eventually cause trees to die. To date, there has been no report of consistent symptoms on the leaves (Facelli *et al*., 2005). Symptoms have been observed mainly on the cultivars Sirora and Kerman, but not on commonly used rootstocks (*P. terebinthus*, *P. atlantica* and *P. integerrima*) (Facelli *et al.*, 2002; 2005). Similar symptoms have been observed on mature female and male trees. The bacterium colonises and blocks the xylem tissue, and may reduce water conductivity (Taylor *et al*., 2005; Sedgley *et al.*, 2006).

Initial investigation of diseased trees showing dieback symptoms led to the isolation of *Xanthomonas* spp. and a potentially pathogenic fungus, *Verticillium tricorpus* (Edwards & Taylor, 1998). Further work by Facelli *et al*. (2002; 2005) confirmed *X. translucens* as the likely cause of pistachio dieback. The bacteria were found consistently in the internal tissue of twigs sampled from affected trees throughout pistachio orchards in Red Cliffs, Robinvale, Renmark, Loxton, Kyalite and Paringa (Taylor & Edwards, 2000). The bacteria have not been found consistently on any external surfaces of affected trees (Sedgley *et al*., 2006; Facelli *et al*., 2009).

Through rep-PCR (repetitive extragenic palindromic polymerase chain reaction), GC-FAME (gas chromatography of fatty acids methyl esters) and SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) analyses, two genetically distinct groups of *X. translucens* were identified to be associated with the disease. The groups were designated A and B (Marefat *et al*., 2006a; Marefat *et al*., 2006b) and were later established as *X. translucens* pv. *pistaciae* A and B through DNA/DNA hybridisation, screening of integrons and *gyrB* gene phylogeny (Giblot-Ducray *et al*., 2009). Group A strains were isolated from diseased plant material collected from all four regions sampled (Kyalite, Renmark, Red Cliffs and Robinvale), whereas group B was only found at Robinvale (Scott *et al*., 2009). So far, the two groups have not been found in one tree (Marefat *et al*., 2006b; Scott *et al*., 2009).

The standard method of disease diagnosis is isolating the pathogen from twigs showing xylem discolouration. After surface sterilisation, the twigs are soaked in sterile distilled water over-night, and the resulting suspensions are streaked on sucrose peptone agar (Moffet & Croft, 1983; Facelli *et al*., 2002; Facelli *et al*., 2005). The pathogen produces characteristic yellow mucoid colonies on this medium. This method usually takes several days to complete and does not allow differentiation of the two groups of *X. translucens*. Marefat *et al*. (2006a) developed a PCR-based technique to improve the detection of the pathogen. This technique is more sensitive and quicker in detecting the presence of *X. translucens* in plant materials than the conventional method. It can also specifically distinguish groups A and B. The sensitivity of this technique was then improved by quantitative PCR (Scott *et al*., 2009). This gives an advantage especially in detecting the pathogen in asymptomatic infected trees, and thus may assist in minimising the spread of the pathogen.

1.4 Management of pistachio dieback

Generally, once a tree is infected with a bacterial pathogen, it is very difficult to eradicate it. Plant pathogens can spread from diseased trees to healthy trees by various means, such as through rain splash, irrigation water, wind, insects, humans or farm tools. In the case of pistachio dieback, *X. translucens* can be spread through infested pruning tools (Sedgley *et al.*, 2006) although the natural means of spread has not been determined. It appears to be localised within the tissue present at the time of inoculation, with minimal or no lateral spread (Taylor *et al*., 2005; Sedgley *et al.*, 2006). To minimise the spread of the pathogen, it is important to practise strict hygiene

measures in orchards affected by the disease. This involves disinfecting pruning tools with bactericide. In trees already affected by the disease, severe pruning with simultaneous application of quaternary ammonium bactericide (Niproquat®) on the pruning wound has been examined as a means to restore the trees to productivity and has shown promising outcomes (Sedgley *et al*., 2006; Taylor *et al*., 2007; Scott *et al*., 2009). The bactericide, however, is not registered for this purpose and the high concentrations required would be costly. The application of copper biocides, standard antibacterial compounds, at various times after pistachio trees were inoculated with the pathogen did not prevent infection. For prevention of the disease, a source of pathogen-free propagation material has been recommended (Sedgley *et al*., 2006). However, low levels of *Xtp* were detected in wood from healthy trees planted at one site proposed as a repository (Scott *et al*., 2009). *Xtp* was not detected in buds collected from infected trees, therefore propagating trees through budding might be considered safe provided that buds were obtained from healthy trees (Scott *et al*., 2009).

Several other studies on the control of pistachio dieback have also been conducted. Antibiotics such as rifamycin at 20 and 10 mg/ml, ampicillin at100 and 50 mg/ml, and streptomycin at 1.0, 0.1 and 0.05 mg/ml, were found to inhibit the growth of *X. translucens in vitro*, and among these three, streptomycin was more effective in inhibiting the pathogen (Taylor & Edwards, 2000). The use of antibiotics, however, is not permitted in the field situation. Foliar application of phosphorous acid for 2 years was noted to reduce the number of new lesions in affected trees but failed to eradicate the pathogen from infected trees (Taylor & Edwards, 2000). Continuous application of phosphorous acid may also result in the chemical residues in pistachio nuts.

The research carried out on management of pistachio dieback has focused mainly on cultural practices in the orchards and use of certain chemicals. While encouraging results have been obtained from these studies, further research is still needed. The use of phosphorous acid, for an example, has demonstrated that this chemical can be used to control the severity of the disease. It is, however, important to test the efficacy of this chemical over several years of application. In addition to this, the use of biological control should be explored as part of a comprehensive integrated disease management program. A basic study on biological control of pistachio dieback should be initiated to investigate the potential of this approach. This information may provide the pistachio industry with another option to manage dieback.

1.5 Biological control

In recent years, biological control has received considerable attention, perhaps due to the increasing consumer preferences for pesticide-free food, and also because of public awareness of the potential cumulative effect of pesticides in the environment. Biological control is the control of plant diseases, pests and weeds by other living organisms (Trigiano *et al*., 2004). Biological control of plant diseases usually involves interaction between the antagonist, pathogen and host.

Several mechanisms, classified as competition, antibiosis and exploitation, may be involved in biological control (Baker, 1968). Competition in the relationship between the antagonists and the pathogen is the common demand by these organisms for an available nutrient supply (Shoda, 2000) and, sometimes, it also involves competition for space to grow. Antibiosis is the production of substances by one microorganism that inhibit or kill another microorganism (Baker, 1968). These substances might comprise compounds that are effective against a wide range of microorganisms or compounds that are selective. Antibiotics are an example of the first group and bacteriocins belong to the second group of compounds (Fravel, 1988; Trigiano *et al*., 2004). Exploitation is parasitism or predation, where the antagonists invade or consume the pathogen (Shoda, 2000). This mode of action has frequently been documented for fungi of the genus *Trichoderma*, which are antagonistic to a wide range of plant pathogenic fungi and bacteria (Okigbo & Ikediugwu, 2000; Ubalua & Oti, 2007). The hyphae of *Trichoderma* grow towards a chemical stimulus produced by pathogens, coil around the hyphae of some pathogens and destroy them (Baker, 1968). This mechanism may be useful in preventing root diseases or reducing inoculum of sclerotium-forming fungi.

Several mechanisms usually occur simultaneously. Sometimes, one mechanism may be active against one specific type of pathogen and another mechanism against another pathogen (Trigiano *et al*., 2004). The ultimate result is reduction in amount of inoculum and spread of the pathogen and in disease severity.

Research and development of microorganisms as biological control agents has been conducted for many decades, but most focus has been on fungi, especially species of the genus of *Trichoderma*. The potential of *Trichoderma* spp. was first recognised by Weindling (1932), when he found the hyphae of *T. lignorum* destroying those of *Rhizoctonia solani* in dual culture. He also found that *T. lignorum* parasitised several

other pathogenic fungi and fungus-like pathogens such as *Phytophthora parasitica*, *Pythium* spp., *Rhizopus* spp. and *Sclerotium rolfsii* (Weindling, 1932). Since then, *Trichoderma* has been used successfully in the biological control of a number of fungal and bacterial plant diseases (e.g. Askew & Laing, 1994; Etebarian *et al.,* 2000; Alfano *et al*., 2007). For example, *T. harzianum* isolate T39 and *T. virens* DAR 74290 reduced the severity of pink rot caused by *Phytophthora erythroseptica* (Etebarian *et al.*, 2000) and *T. hamatum* 382 provided protection against soft rot of tomato caused by *Xanthomonas euvesicatoria* by inducing systemic changes in the physiology and disease resistance of the plants (Alfano *et al*., 2007).

Of 11,000 plant diseases, about 70% are caused by fungi (Agrios, 2005). It has been estimated that fungal plant pathogens include species from 120 genera, and other causal microorganisms belong to eight genera of bacteria and 30 types of viruses (Ferreira *et al*., 2006). Given the importance of fungal diseases in plants, the use of bacteria as biological control agents is much directed towards controlling fungal pathogens, particularly soil-borne and foliar pathogens. Bacteria shown to have potential as biological control agents are from many genera, including *Agrobacterium* (Li *et al*., 2009; Wei *et al*., 2009), *Bacillus* (Korsten *et al*., 1997; Jock *et al*., 2002; El-Hassan & Gowen, 2006; Lee *et al*., 2008; Zhang *et al*., 2008b), *Erwinia* (Vanneste *et al.*, 1992; Kearns & Mahanty, 1998; Wright *et al*., 2001), *Pseudomonas* (Loper *et al*., 2007; McSpadden Gardener, 2007), *Streptomyces* (Crawford *et al*., 1993; Neeno-Eckwall *et al*., 2001; Schottel *et al*., 2001; Errakhi *et al*., 2007; Bressan & Figueiredo, 2008) and *Xanthomonas* (Kobayashi *et al*., 1995; Moss *et al*., 2007). Many of these have been evaluated *in vitro*, some were applied effectively in field trials and a number of them have been developed commercially. Of the six genera of bacteria listed above, *Bacillus* and *Pseudomonas* are the most appealing candidates to be evaluated further as biological control agents for dieback disease in pistachio. Both bacteria have been reported as endophytes (Lodewyckx *et al*., 2002), therefore, are able to grow in the vascular tissue of plants, and these genera represent the different types of bacteria; *Bacillus* being Gram-positive and *Pseudomonas* Gram-negative.

1.5.1 Use of *Bacillus* spp. in biological control

Among the *Bacillus* spp. well-researched in control of fungal and bacterial diseases are *B. amyloliquefaciens, B. cereus, B. megaterium, B. pumilus* and *B. subtilis* (Jock *et al*., 2002; Wulff *et al*., 2002a; Yu *et al*., 2002). *B. amyloliquefaciens, B.*

pumilus and *B. subtilis* showed antagonistic activity against *Xanthomonas campestris* pv. *campestris*, the causal pathogen of black rot in cabbage (Wulff *et al.* 2002a). When cabbage plants grown from seeds inoculated with the antagonists were examined 20 days after germination, the three *Bacillus* species evaluated were found to have colonised cabbage seedlings with different efficacy. *B. pumilus* showed the best endophytic ability, with isolates BF3 and 60 recovered from all the plant parts sampled (roots, stem, cotyledon and true leaf) and the second was *B. subtilis,* with isolates 77 and 7D re-isolated mainly from roots and less frequently from the stem sections. *B. amyloliquefaciens* showed the least endophytic ability to colonise the cabbage seedlings. The three *Bacillus* spp. reduced the incidence of black rot in the seedlings, but the efficacy of the isolates was also affected by the weather and soil conditions (Wulff *et al.*, 2002b).

Most studies on *Bacillus* spp. have focused mainly on their antagonistic activity through production of secondary metabolites such as antibiotics, volatile organic compounds, antifungal, antiviral, insecticidal and immunosuppressant agents. Some of these metabolites suppress the growth of plant pathogens. Different species of *Bacillus* produce many types of antibiotics. *B. subtilis*, for example, produces fengycins, iturin A, lipopeptides, subtilin, subtilosin and surfactins (Nagórska *et al*., 2007). *B. amyloliquefaciens*, very closely related to *B. subtilis,* was found to produce an antifungal compound, iturin A, which inhibits the growth of *Rhizoctonia solani in vitro* (Yu *et al.*, 2002). Several strains of *B. subtilis* were also reported to inhibit the growth of *Xanthomonas oryzae*, the causal agent of bacterial blight of rice *in vitro* by secreting antimicrobial peptides (Lin *et al*., 2001; Wu *et al*., 2009). The gene encoding a peptide extracted from *B. subtilis* strain SO113 was successfully cloned into and expressed in another *B. subtilis* strain, suggesting the possibility of using genetic engineering of disease resistance in rice through transgene expression (Lin *et al*., 2001).

As well as producing antibiotics, *B. subtilis* is known to produce siderophores (Marten *et al*., 2000). Siderophores act as iron chelators thus making iron, a compound important for metabolism, unavailable to other microorganisms (Lemanceau *et al*., 2009), and this would subsequently retard the growth of pathogenic bacteria. Production of siderophores is an example of competition in the antagonist-pathogen relationship (Lemanceau *et al*., 2009). The ability of *Bacillus* spp. to colonise plants endophytically and exclude pathogens is also classified under this mechanism (Ryan *et al.*, 2008).

The ability of *Bacillus* species to colonise the internal tissues of plants, to produce metabolites to suppress plant pathogens and to inhibit pathogenic *Xanthomonas*, suggests that members of the genus may have potential in the biological control of pistachio dieback.

1.5.2 Use of *Pseudomonas* spp. in biological control

Pseudomonas spp., Gram-negative and rod-shaped bacteria, have been documented to suppress a variety of soil-borne bacterial plant pathogens (Mercado-Blanco & Bakker, 2007; Trivedi *et al*. 2008). One of the best studied species is *Pseudomonas fluorescens*, which has been reported to prevent the rotting of potato tubers caused by *Erwinia carotovora* (Burr *et al*., 1978; Cronin *et al*., 1997) and to suppress bacterial blight of rice (*X. oryzae*) in field experiments (Velusamy *et al*., 2006).

Many strains of *Pseudomonas* have been isolated from soils microcosms, but some have also been found as endophytes, living in the stem tissue of plants. *P. fluorescens* is the major antagonistic endophytic bacterium isolated from eggplants (Ramesh *et al*., 2009) and *P. putida,* originally isolated from healthy cotton plants, suppressed the disease expression of Fusarium wilt of cotton (Chen *et al.*, 1995). In terms of woody trees, *Pseudomonas* spp. were isolated from the stems of lemon (Gardner *et al*., 1982) and coffee (Vega *et al*., 2005). *Pseudomonas* spp., mostly *P. corrugata,* were also isolated from the xylem sap of grapevines (Bell *et al*., 1995). The endophytic *Pseudomonas* isolated from the host plants often do not incite disease (Mercado-Blanco & Bakker, 2007). The above authors evaluated these isolates as biological control agents and have found that they were effective in suppressing the growth of pathogen. However, *Pseudomonas* so far has not been evaluated for ability to control diseases caused by *Xanthomonas* spp..

Production of antibiotics, enzymes and siderophores has been associated with the ability of *Pseudomonas* spp. to suppress plant pathogens and disease. An antibiotic, 2,4-diacetylphloroglucinol (DAPG), produced by *P. fluorescens* strain F113 inhibited the growth of *E. carotovora* subsp. *atroseptica* on wounded potato (Cronin *et al*., 1997) and also *Pseudomonas* sp. strain EB67 inhibited *Ralstonia solanacearum in vitro* (Ramesh *et al*., 2009). As well as DAPG, a different strain of *P. fluorescens* also produced the antibiotic pyrrolnitrin which suppressed the growth of fungal pathogens

Thielaviopsis basicola, *Alternaria* sp. and *Verticillium dahliae*, but was less effective on *Pythium ultimum* and *F. oxysporum* when evaluated *in vitro* (Howell & Stipanovic, 1979). Pyrrolnitrin is also produced by *P. cepacia,* which inhibited *Rhizoctonia solani* and *V. dahliae in vitro*, but not the bacterial pathogens *E. carotovora*, *P. solanacearum*, *X. campestris* pv. *oryzae*, *Agrobacterium tumefaciens* and *Corynebacterium michiganense* (Homma *et al*., 1989). Other antibiotics produced by *Pseudomonas* spp. are phenazine carboxylic acid (Gurusiddaiah *et al*., 1986) and pyoluteorin (De La Funte *et al*., 2004). Most antibiotics from the *Pseudomonas* spp. were extracted from the bacteria, purified and evaluated *in vitro* on the plant pathogens.

P. fluorescens strain A506 is known to produce antibiotic(s) in culture media. However, in an environment with limited availability of iron such as on the surfaces of pear and apple flowers, *P. fluorescens* A506 suppressed *E. amylovora* by producing siderophores and competed for the available nutrients (Temple *et al*., 2004). Siderophores produced by *P. fluorescens* WCS417 and *P. putida* WCS358 were found to trigger systemic resistance of grapevine to *Botrytis cinerea* (Verhagen *et al*., 2010). Another mode of antagonism exhibited by *Pseudomonas* is production of volatile metabolites. *P. corrugata* inhibited the growth of *A. alternata* and *F. oxysporum*, as well as bacterial wilt pathogens of many plant species, *in vitro* on tryptone yeast extract agar, by production of ammonia, a volatile metabolite (Trivedi *et al.*, 2008).

As *Pseudomonas* spp. can colonise the internal tissue of woody plants and suppressed bacterial diseases, species of this genus may also have potential in biological control of pistachio dieback.

1.5.3 Commercial bacterial biological control agents

Several bacteria have been successfully developed into commercial formulations. To illustrate the need to find suitable formulations for control of pistachio dieback, the following is focused on commercial biological control of bacterial diseases, such as fire blight of pome fruit.

Pseudomonas syringae strains ESC10 and ESC11, originally isolated from apple trees, were developed commercially as Bio-Save 10LP® and Bio-Save 11LP®, respectively, to prevent postharvest diseases of citrus, pome and stone fruits and potatoes (Stockwell & Stack, 2007). BlightBan®, developed from *P. fluorescens* strain

A506, is a registered biopesticide for suppression of fire blight caused by *E. amylovora* in apple and pear trees (Johnson & Stockwell, 1998). The strain was most effective in suppressing *E. amylovora* when applied 72 hours before inoculating apple flowers with the pathogen (Wilson & Lindow, 1993). As this particular strain of *P. fluorescens* did not exhibit antibiosis against *E. amylovora in vitro*, the primary mode of antagonism was believed to be competitive exclusion. When compared separately, *P. fluorescens* multiplied at the same rate in inoculated pistils, but when co-inoculated with the pathogen, *P. fluorescens* was unable to compete for the limited nutrients available, and therefore could not prevent the pathogen from colonising the blossom. When inoculated 72 hours before the pathogen, *P. fluorescens* suppressed the population of *E. amylovora* to 10^3 cells/ml, a concentration which was unlikely to initiate infection in the floral tissues of the tree. *P. fluorescens* A506 in BlightBan® is formulated as lyophilised cells and requires low temperature for storage (Johnson & Stockwell, 1998).

In terms of formulation, *Bacillus* spp. offer many advantages over Gram-negative bacteria. The resistance of *Bacillus* endospores to heat and desiccation allows them to be easily formulated and stored (Emmert & Handelsman, 1999). Some examples of commercial formulations based on *B. subtilis* are Serenade®, Rhapsody®, FZB24®, and Biopro® (Brannen & Kenney, 1997; Paulitz & Bélanger, 2001; Broggini *et al.*, 2005). Most products are intended to suppress fungal diseases but Serenade® and Biopro® have been registered for fire blight, caused by *Erwinia amylovora* (Broggini *et al.*, 2005). The direct application of Biopro® containing 2 x 10^6 spores of *B. subtilis* /ml on apples flowers during blooming stage, resulted in colonisation of the pistils by the biological control agent (*B. subtilis)* (Broggini *et al.*, 2005). Colonisation of the open flower by *B. subtilis* following application of Biopro® to flowers that were closed or at the bud stage (termed secondary colonisation) was influenced by environmental conditions at the time of application. Foraging honeybees were reported to assist in the secondary colonisation of blossoms by the biological control agents (Maccagnani *et al*., 2009). At low temperature, the exposure time of the opened flowers to the disseminators was short, therefore reducing the population size of *B. subtilis* on the blossoms and affecting the colonisation of the flowers by the biological control agent.

The above articles document the success of biological control agents in commercial formulations, evaluated in field conditions. The effect of these commercial bacterial-based products on *Xanthomonas* spp., however, has not been tested.

1.5.4 Application of biological control agents into the plant system

For systemic plant diseases such as dieback and wilts of woody species, a technique to introduce the biological control agents into the vascular system of the plant is considered important. The antagonists need to survive well and grow to suppress or reduce the population of the pathogen. Various methods have been studied, among those documented were spraying (Scherm *et al*., 2007), injection (Chen *et al*., 1995), direct inoculation to the wounded surface of plant (Cronin *et al*. 1997), or adding the inoculum suspension into the soil (Berg *et al*., 2001; Paulitz & Bélanger, 2001; Stewart, 2001). Introducing the antagonists to the plant via the root system would be difficult. Before the antagonists can colonise the roots of the plants effectively, they need to compete with indigenous microflora for nutrients and should be able to tolerate the physical and chemical conditions of the soil environment (Albareda *et al*., 2006). This technique has been successfully applied in control of some soil-borne diseases, such as Verticillium wilts (Uppal *et al*., 2008; Giotis *et al*., 2009) and Fusarium wilts (Israel *et al*., 2005; Zhang *et al*., 2008a), but seldom to other vascular plant diseases involving above-ground plant parts, such as dieback of woody plants caused by *Eutypa lata* (Ferreira *et al*., 1991).

Direct inoculation of biological control agents onto the surface of plant tissue has been frequently used to evaluate the efficacy of antagonists in suppressing the growth of pathogens of woody plants (Munkvold & Marois, 1993; Chapuis *et al*., 1998; Schmidt *et al*., 2001a; John *et al*., 2005). Most such techniques involve introducing the biological control agents to the plant tissue before challenge with pathogen. Biological control agents evaluated were either introduced microorganisms (John *et al*., 2004; John *et al*., 2005; Schubert *et al*., 2008) or those originally isolated from the host plant (Ferreira *et al*., 1991; Munkvold & Marois, 1993; Chapuis *et al*., 1998). The effect of the antagonists on the plant pathogens was evaluated by sampling and cutting the inoculated wood into small discs, and assessing the growth of both antagonist and pathogen on specific culture media. The percentage of wood samples colonised by antagonist or pathogen was obtained by visual identification of the fungal or bacterial colonies growing from the wood discs. This method may be sufficient to measure the relative effect of antagonist and the controls, but is not adequate to compare the population of antagonist or pathogen after treatments.

The application of biological control agents described above generally was associated with treatments to pruning wounds. In commercial pistachio orchards, pruning is conducted, usually in winter, to manage the distribution of vegetative growth on the tree, making the tree accessible for mechanical harvesting and pest management (Spann *et al*., 2008a). Through pruning also, *Xtp* can be spread if the tools are contaminated with the pathogen (Sedgley *et al.*, 2006). Therefore, an approach of combining the application of biological control agents to healthy trees (as described above) and the existing pruning programme might be appropriate.

From the initial site of inoculation, the biological control agents may remain localised at the point of inoculation, or spread internally throughout the plant (Andreote *et al*., 2009). Their survival and establishment in the plant system then would depend on the genotype of the host plant (Reiter *et al*., 2003; Fritschi *et al.*, 2007), growth temperature (Whipps *et al*., 2008) and the presence and interaction with the plant pathogen (Araújo *et al*., 2002). Biological control agents that can colonise the plant tissue may be able to provide effective control of the dieback pathogen.

1.5.5 Use of antimicrobial peptides in biological control

Besides the siderophores, bacteria produce two important categories of antimicrobials; the antibiotics and bacteriocins (Holtsmark *et al*., 2008). Antibiotics have been used in the management of plant diseases since the 1950s (McManus *et al*., 2002). They can be effective, but are expensive, and resistance to the antibiotic may occur in plants. The other major obstacle to using antibiotics to control plant disease is that this is not permitted in Australia or most European countries because of the risk of transfer of antibiotic resistance genes into human pathogens. In contrast, bacteriocins are not likely to confer antibiotic resistance or affect human health and may, therefore, represent a good option. Bacteriocins also would have low negative impact on the environment and be selective for certain bacteria (Badosa *et al*., 2007).

Bacteriocins are usually biochemically characterised by their structures and mechanisms of actions and are classified into four distinct classes (Klaenhammer, 1993). Bacteriocins in Class I and Class II result in the destabilisation of the membrane, pore formation and inhibition of cell wall synthesis of microorganisms including plant pathogenic bacteria, whereas those in Class III lyse bacterial cells (Holtsmark *et al*., 2008). Through these mechanisms, bacteriocins affect the growth of microorganisms.

Class IV bacteriocins are more complex and carry essential lipids and the cyclic peptides (Klaenhammer, 1993).

Antimicrobial peptides (AMPs) are produced by animals, insects, plants and a wide range of microorganisms such as bacteria, and usually have long amino acid sequences. The AMPs obtained from living organisms, especially the peptaibols and lipidic cyclopeptides, are very difficult to exploit in plant disease control due to the small amount present and also due to their considerable phytotoxicity (Montesinos, 2007). Therefore, alternative AMPs, have been designed, based on reports of natural AMPs active against plant pathogens, and produced by synthetic or biotechnological methods. These synthetic AMPs are less toxic analogues, composed of shorter amino acids sequences, and are more stable in the normal environment than naturally occurring AMPs (Marcos *et al*., 2008).

Synthetic AMP, D4E1, inhibited the growth of the fungal pathogens *Thielaviopsis basicola*, *V. dahliae*, *F. moniliforme*, and the fungus-like *Phytophthora cinnamomi* and *P. parasitica* (Rajasekaran *et al*., 2001). Peptides PAF26, PAF38, PAF40 and BM0 were able to protect oranges from the post-harvest disease green mould caused by *Penicillium digitatum* (Muñoz *et al*., 2007). Fifteen linear undecapeptides were highly active to inhibit the survival of *F. oxysporum* microconidia, and among them, BP21 and BP34 were sporocidal against *Penicillium expansum*, the causal agent of rot in apples (Badosa *et al*., 2009). Synthetic peptides were also documented to suppress growth of bacteria. As well as fungal pathogens, D4E1 also inhibited growth of *P. syringae* pv. *tabaci* and *X. campestris* pv. *malvacearum* (Rajasekaran *et al*., 2001). Ferre *et al*. (2006) evaluated peptide Pep3 and eleven others, derived from Pep3, to suppress growth of *E. amylovora*, *P. syringae* pv. s*yringae* and *X. vesicatoria* pv. *vesicatoria*. BP09, BP10, BP18, BP19 and BP76 at 2 μM completely inhibited the growth of *X. vesicatoria in vitro*, and BP76 was highly bactericidal against the pathogen after 90 minutes of exposure at a concentration of 5 μM. "Bactericidal" indicates that the peptide killed the pathogen cells and, hence the effect of the compound is longer lasting. If the inhibitory effect occurs only when the compound is present, it is defined as "bacteriostatic" (Montesinos & Bardají, 2008). Based on peptide BP76, Badosa *et al*. (2007) designed and synthesised another 125 peptides, and evaluated these peptides against the same group of bacterial pathogens used by Ferre *et al*. (2006). *X. vesicatoria* was more sensitive to all the peptides tested than were the other two bacteria. Peptide BP100 was the best among five peptides evaluated *in vivo*

against *E. amylovora* at 100 and 200 μM. This peptide reduced disease incidence by 63% in detached apple flowers and 74% in pear flowers. However, the evaluation of peptides by these authors did not address the effect of peptides on different inoculum concentration of the pathogens, nor their effect on different pathogen isolates. Also, the efficacy of peptides to inhibit growth of plant pathogens in living trees has not been evaluated.

The role of AMPs in suppressing the growth of bacterial plant pathogens highlighted above is useful as a base to study the efficacy of peptides against *X. translucens in vitro*.

1.6 Summary and project aims

Effective methods for control of dieback, a bacterial disease affecting pistachio in Australia, are lacking. The success in biological control of plant pathogens and diseases documented above indicates the potential of this approach as a tool in managing pistachio dieback. Although most biological control agents used in suppressing the growth of plant pathogens are fungi, there is also evidence of bacteria used to control bacterial plant diseases. Bacteria belonging to the genera *Bacillus* and *Pseudomonas* might have potential for further testing as both are well-studied and have been reported to suppress bacterial pathogens. Furthermore, the ability of both bacteria to grow as endophytes may allow them to reduce the colonisation of plant tissues by pathogens.

An understanding of the mechanisms involved in the biological control of disease is important. The main mechanism usually associated with *Bacillus* and *Pseudomonas* is production of antimicrobial metabolites with antibiotic properties. The ability of antagonists to exert antimicrobial effects on or in host plants needs to be established. The method of application of antagonists to protect healthy trees from infection also needs to be investigated to identify practical methods for implementation.

Advanced technologies have allowed peptides and small proteins to be extracted from microorganisms or plants for use in controlling disease. Even though peptides have not been used in the field, the potential of synthetic peptides to suppress growth of plant pathogens has been demonstrated *in vitro*. Thus, the potential of synthetic peptide to control *Xtp* merits further investigation.

The overall aim of this study was to investigate the potential of bacteria in the biological control of pistachio dieback. The objectives were to

1. Assess the ability of eight bacteria isolated from pistachio wood to suppress growth of *Xtp in vitro*.
2. Investigate the potential of bacterial antagonists to reduce the colonisation of pistachio wood by *Xtp.*
3. Assess the ability of peptide BP100 to inhibit the growth of *Xtp in vitro*.

CHAPTER TWO

General materials and methods

2.1 Potential antagonists and pathogen

Eight isolates of bacteria were screened for their ability to inhibit growth of *Xtp in vitro*. *Bacillus subtilis* was included in this study since the species has been well-studied and reported to inhibit several Gram-negative plant pathogenic bacteria *in vitro* (Jock *et al*., 2002; Hammami *et al.,* 2009). The *B. subtilis* isolate used in the experiments reported here was obtained from the teaching unit, School of Agriculture, Food and Wine, University of Adelaide. Seven other isolates, not previously identified, were tested also, namely 64161-7, 71164-16, SUPP, CBP, PC506, PC507 and PC397. These had been isolated by Evelina Facelli and Cathy Taylor from pistachio trees and stored following indications that they may antagonise *X. translucens*. All isolates were grown in sucrose peptone broth (SPB) (Facelli *et al*., 2002) supplemented with glycerol and stored at -80^{o}C.

The pathogen used in all the experiments was *X. translucens* from Group A (isolate DAR75532) (Giblot-Ducray *et al*., 2009), which was isolated from a diseased pistachio tree in Kyalite, New South Wales (Facelli *et al*., 2005). The isolate was also grown in SPB supplemented with glycerol and stored at -80^{o}C.

2.2 Revival of potential antagonists and pathogen

At the beginning of every experiment, pathogen and antagonists were retrieved from -80^{o}C and transferred to sucrose peptone agar (SPA). Cultures were incubated at 28^{o}C in the dark for 72 hours before use. Suspensions of DAR75532 for use in all experiments were prepared by transferring two loopsfull from the resulting colonies to tubes containing 10 ml SDW or, in some experiments, SPB was used instead of SDW. The tubes were shaken gently to ensure that the bacteria were well distributed in the suspension. The number of colony forming units (CFU) in 1 ml of DAR75532 suspension was determined with the aid of haemocytometer, and the suspension was adjusted to 10^6 CFU/ml by serial dilution. Aliquots of 100 µl were spread on SPA and incubated at 28^{o}C in the dark. From 48 hours of incubation up to another 14 days, the

plates were assessed to confirm that the suspensions contained viable cells of DAR75532.

The concentration of the antagonists was not determined. However, two loopsfull of antagonist were taken from a 72-hour old culture and diluted ten-fold in SDW, for use in the screening.

2.3 Culture media

Two types of media were used throughout the experiments; sucrose peptone agar (SPA) and nutrient agar (NA) (See Appendix 1). SPA was selected as this medium was reported as suitable for growing *X. translucens* (Facelli *et al*., 2002) and NA is a general medium for growing bacteria (Trigiano *et al*., 2004). Approximately 20 ml of media were dispensed into each Petri dish of 90 mm diameter.

2.4 Agar diffusion technique

A method adapted from Parente *et al*. (1995) was used in the preliminary screening of the potential antagonists. One hundred µl of DAR75532 suspension were spread on SPA and NA plates and left to dry in the laminar flow cabinet for about an hour. When the surface of the agar had dried, one well with 6 mm diameter was punched aseptically in the centre of each plate. Then, the wells were filled with 20 µl of the antagonist suspension. The plates were left at room temperature for 1 to 2 hours to allow the suspension to diffuse into the medium, before incubating at 28^{o}C in the dark. This method was also used to evaluate the inhibitory effect of metabolites of the potential antagonists on the growth of DAR75532.

2.5 Measurement of inhibition zone

Where growth of DAR75532 was inhibited by antagonists, the inhibition zone was measured 48 hours after the introduction of the antagonists in the well. Four measurements from the edge of the well to the edge of the inhibition zone were taken perpendicular to each other. The four measurements were averaged to give a final measurement of the inhibition zone (Yilmaz *et al*., 2006) (see Figure 2.1).

2.6 Statistical Analysis

Where appropriate, experiments were arranged in a Randomised Complete Block Design (RCBD). Data obtained from the experiments were analysed using GENSTAT for windows, 10th edition (Lawes Agricultural Trust, Rothamsted, England).

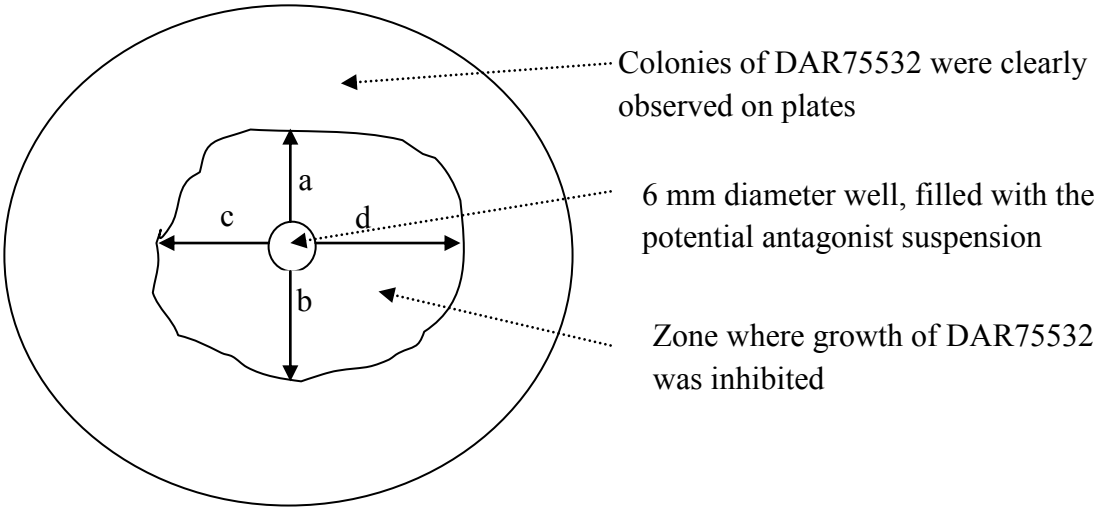


Figure 2.1: The degree of antibacterial activity of the antagonists was assessed by the radius of inhibition zone on the medium. Four measurements (a, b, c and d) were taken perpendicular to each other, from the outer edge of the well to the edge of the clear zone. The four measurements were averaged to give a final measurement of inhibition zone.

CHAPTER THREE

Preliminary study of antagonism and characterisation of potential antagonists

3.1 Introduction

One approach in developing biological control for a plant disease is to isolate microorganisms and screen isolates for potential to suppress the growth of pathogen. The preliminary screening is often done *in vitro* and a standard assay needs to be developed to assess the antagonistic ability of the selected microorganism. There are several well-established protocols for screening potential bacterial antagonists, but these are mostly used in screening for activity against fungal pathogens. A popular assay involves the dual culture technique, which usually involves the use of mycelium plugs of the appropriate fungal pathogen, placed in the middle of a plate containing a culture medium mixed with the antagonist(s) being tested (Leelasuphakul *et al.*, 2008). Other techniques involve placing the mycelium plug at the edge of the plate and spreading an aliquot of known concentration of antagonist at the other side of the plate (Basha & Ulaganathan, 2002; Rajkumar *et al.,* 2005). The antagonistic ability of the bacterial isolates is then determined by measuring the inhibition zones produced on the medium.

The agar diffusion assay is also frequently used in screening the potential of bacteria to antagonise bacterial pathogens. Here, the susceptibility of the pathogen is detected by challenging it with suspension of the antagonist that is placed in a well in an agar plate that has been seeded with a lawn of pathogen bacteria. As described above, the antagonistic ability of the bacteria is determined by the inhibition zones produced. The advantage of this assay is that it is easy to use and reliable in quantifying the antimicrobial activity of the antagonists (Parente *et al*., 1995). The extent of the inhibition zones produced on the plates is relatively easy to measure.

Once a microorganism has been shown to inhibit growth of one particular pathogen, the mechanism contributing to the suppression of the pathogen‟s growth may be determined. The modes of antagonism usually documented are antibiosis, parasitism and competition (Baker, 1968) as described in section 1.5. Antibiosis through production of volatile and diffusible metabolites, and competition for nutrients were investigated in the experiments reported in this chapter. The inverted plate technique

(Singh *et al.*, 2005) was used to examine the production of volatile metabolites active against DAR75532 whereas the diffusible metabolites were examined by the agar diffusion assay (Parente *et al*., 1995). This was later extended by examining the production of antibiotics in broth culture. Competition for nutrients between the antagonists and DAR75532 was studied by growing both bacteria in the same liquid medium for a period of time (Cronin *et al*., 1997). The aims of the experiments were to screen potential antagonists for ability to inhibit the growth of DAR75532 and to study the mechanisms that might be involved. Preliminary characterisation of the antagonists is also reported.

3.2 Materials and Methods

3.2.1 *In vitro* screening

DAR75532 was prepared in SDW or SPB as described in section 2.2. In this preliminary experiment, various concentrations of DAR75532 were examined. At a concentration of 10^4 CFU/ml, DAR75532 did not grow uniformly on SPA. In contrast, the growth resulting from suspensions of 10^7 and 10^6 CFU/ml was consistent on both SPA and NA. The 10^6 CFU/ml concentration was selected for use in subsequent experiments.

The screening was conducted following a method adapted from Parente *et al*. (1995) as described in section 2.4. Each antagonist was tested separately by pipetting 20 µl of the diluted suspensions into the wells in NA and SPA and plates were incubated at 28^{o}C in the dark. The inhibition zone produced on the agar was measured 48 hours later. Each antagonist and medium combination was replicated five times. Controls were composed of SDW in the wells, instead of the antagonist suspensions. After the measurements were recorded, to determine if the effect was bactericidal or bacteriostatic, the surface of SPA in the inhibition zones was scraped with an inoculating loop and streaked on fresh SPA. The plates were then incubated at 28^{o}C in the dark and growth of DAR75532 was assessed daily from 48 hours up to 14 days.

3.2.2 Volatile metabolite assay

The ability of the potential antagonists to produce volatile metabolites inhibiting the growth of DAR75532 was investigated through an inverted plate assay (Singh *et al*.,

2005). A suspension of DAR75532 containing 10^6 CFU/ml was prepared as in section 2.2. One hundred µl of the suspension were spread on either SPA or NA. One hundred µl of antagonist suspension containing approximately 10^6 CFU/ml were also spread, separately, on SPA or NA plates. About half an hour later, plates with DAR75532 were inverted over the bases of plates containing antagonists, in a laminar flow cabinet. The plates were sealed with masking tape to prevent leakage of possible volatile metabolites produced by the antagonists. Plates were then incubated in the dark at 28°C and inspected daily for growth of DAR75532. Positive controls consisted of plates with DAR75532 culture inverted over plates spread with SDW and negative controls were plates spread with SDW inverted over plates containing antagonists. All treatments were replicated four times.

3.2.3 Antibiotic activity assay

All the selected antagonists were tested for antibiotic activity of cell-free culture filtrate (CFCF) against DAR75532 on both NA and SPA. To ensure that the volume of medium used did not have any effect on the results of the experiment, each Petri dish was filled with 20 ml of culture medium.

3.2.3.1 Preparation of cell-free culture filtrate

Antibiotic production by the antagonists was assessed using a method described by Mari *et al*. (1996). The antagonists were first retrieved from -80°C and suspensions in nutrient broth prepared as described in section 2.2. The tubes were then placed on a shaker at 150 rpm at 28°C. After 24 hours, the bacterial cells were pelleted by centrifugation at 10,000 g for 20 minutes and the supernatants were then filtered through 0.45 µm Millipore MF membrane filters (Millex-HA) to yield CFCF. These CFCF were used immediately in the experiments described below.

3.2.3.2 Inhibition of DAR75532 by CFCF

An agar well diffusion assay similar to that described in section 2.4 and a paper disc technique (Charoensopharat *et al*., 2008) were used to examine antibiotic activity of the antagonists. In the agar diffusion technique, 20 µl of CFCF from each antagonist separately, were administered to the wells and allowed to diffuse into the agar for 1 to 2 hours before the plates were incubated in the dark at 28°C. In the paper disc technique,

paper discs (Whatman No 1 filter paper) with diameter of 6 mm were autoclaved at 120^{o}C for 20 minutes before use. The paper discs were impregnated with 20 µl of CFCF before placing one in the centre of each plate. Negative controls consisted of 10 µl SDW in the well or on the paper disc. Antagonist suspensions without centrifugation and filtration served as positive controls. All treatments were replicated five times and incubated in the dark, at 28^{o}C. The inhibition zones of DAR75532 on the agar were recorded daily from 48 hours until 14 days.

3.2.3.3 Production of diffusible metabolites by *B. subtilis*

Results of the *in vitro* screening experiment suggested that the antagonists produced diffusible metabolites into the medium. However, the results obtained with the CFCF indicated otherwise, prompting experiments to determine optimal conditions for metabolite production. The strain of *B. subtilis* was selected because this species is known to produce antibiotics inhibitory to growth of various plant pathogens (Asaka & Shoda, 1996; Leelasuphakul *et al*., 2006). The incubation time, centrifugation speed and time, and volume of CFCF were modified to assess the effect of such changes on the ability of CFCF to affect growth of DAR75532. The first experiment involved 24, 48 and 72 hour incubation times for *B. subtilis* to produce the metabolites. The second experiment consisted of two centrifugation speeds and times for removing the bacterial cells; 10,000 g for 20 minutes (Mari *et al*., 1996) and 3,000 g for 10 minutes (Wulff *et al*., 2002a). In the third experiment, three different amounts of CFCF were used in the agar diffusion assay; 20, 30 and 40 µl. In the second and third experiments, the incubation time of *B. subtilis* was 48 hours. The agar well diffusion technique as described in section 2.4 was used to detect the effect of the metabolites produced by *B. subtilis* against DAR75532. Negative and positive controls were SDW and *B. subtilis* suspensions in the well, respectively. Only SPA was used, as the diffusible metabolites were observed to be active on SPA (section 3.1.1). All experiments were replicated four times. The inhibition zones produced on the agar were recorded 48 hours after treatment.

3.2.3.4 Production of diffusible metabolites by *B. subtilis* and isolate 64161-7

Unexpected results obtained from the above experiment prompted another attempt to modify the technique to assess antibiotic production of the antagonists. A method employed by Zhang *et al*. (2008b) was used to examine the effect of diffusible

metabolites produced by *B. subtilis* and isolate 64161-7 on the growth of DAR75532. Isolate 64161-7 was used in this experiment as it inhibited growth of DAR75532 *in vitro*. Ten ml of SPB containing 10^6 CFU/ml of DAR75532 were added to 400 ml of melted SPA cooled down to about 50°C. Twenty ml of the medium was poured into each plate and left for an hour to solidify. CFCF of both *B. subtilis* and isolate 64161-7 were prepared as in section 3.2.3.1 except that the centrifugation speed and time used were 1,000 g for 20 minutes. The agar diffusion assay was used to determine the effect of metabolites produced by administrating 40 µl of CFCF into each well. Supernatant of bacterial cultures after centrifugation but without filtration was also used as one of the treatments. The positive controls consisted of *B. subtilis* or suspension of isolate 64161-7 and the negative control was SDW. Each treatment was replicated three times and the inhibition zones were recorded 48 hours after treatment.

3.2.3.5 Assessment of antibiosis in broth culture

The techniques described above were believed to be insufficiently sensitive to detect the presence of diffusible metabolites. As such, a different technique, suggested by Professor Giora Kritzman (The Volcani Center, Israel) was used. Due to time constraints, only two antagonists could be tested in this experiment. Isolates PC397 and 64161L were selected based on their ability to inhibit DAR75532 *in vitro* as reported in section 3.3.1. Isolate 64161L was derived from isolate 64161-7 and was screened separately against DAR75532 (see section 3.2.5.4).

Isolates PC397 and 64161L were first retrieved from -80°C and grown on SPA for 72 hours. The media used were nutrient broth (NB, Oxoid Ltd., England), nutrient broth supplemented with 0.5% yeast extract (NB+YE) and nutrient broth supplemented with 0.5% yeast extract and 0.5% glucose (NB+YE+G). A volume of 150 ml of each medium was dispensed into each 250 ml Erlenmeyer flask and each flask was inoculated with two loopsfull of the appropriate antagonist. The flasks were then placed on an orbital shaker at 27°C for 7 days. After incubation, the antagonist suspensions were centrifuged at 7,500 rpm for 20 minutes using a Sorval® RC-5B centrifuge (Du Pont Instruments, Australia). The supernatants were filtered through 0.22 µm filters to yield CFCF.

Each CFCF was then added separately to the same fresh, sterile medium, in 50 ml Erlenmeyer flask. Ten ml CFCF were added to 10 ml of medium, and this became

dilution 1:1. From this suspension, 10 ml were diluted again in 10 ml of the same medium producing 1:2 diluted CFCF, and this process was repeated to give 1:4 diluted CFCF. A suspension of DAR75532 was prepared as described in section 2.2 except that sterile 0.085% sodium chloride solution (sterile saline) was used for dilution. One hundred µl of DAR75532 suspension was pipetted into each flask containing the CFCF of 64161L and PC397 at dilutions 1:1, 1:2 and 1:4, and every dilutions were replicated three times. The flasks were then incubated at 27^{o}C for 4 days. Antibiotic activity was considered to have occurred when the suspension remained clear, whereas a turbid suspension indicated growth of DAR75532 and therefore, no antibiotic activity. After 5 days of incubation, 100 µl of aliquots from clear suspensions were spread evenly on SPA and NA supplemented with cephalexin, ampicillin and gentomicin (NA+ab, see Appendix 1). NA+ab was developed by Ms Tu Anh Vu Thanh (pers. comm.., 2009) as a semi-selective medium for isolating *Xtp* from wood buried in soil. These plates were then incubated in the dark at 28^{o}C from 48 hours up to 14 days, and the growth of DAR75532 recorded.

3.2.4 Competition assay

Besides the production of antibacterial metabolites, competition between DAR75532 and the antagonists may occur. Each antagonist was tested for its ability to compete with DAR75532 separately.

The method of Cronin *et al.* (1997) was used in this experiment. DAR75532 at 10^6 CFU/ml was prepared and diluted in SPB as described in section 2.2. Similarly, suspensions of all eight potential antagonists, each at concentration of 10^6 CFU/ml, were prepared separately. Equivalent volumes (3 ml) of DAR75532 suspension and suspensions of each antagonist were mixed in sterile tubes. The combinations of DAR75532 with each bacterial antagonist were replicated four times, and controls comprised either DAR75532 in SPB or antagonist in SPB. The mixtures were then shaken gently to ensure that DAR75532 and the antagonist cells were evenly distributed. The mixtures were incubated at 28^{o}C with continuous shaking at approximately 80 rpm for 48 hours. Then, 100 µl of each suspension were spread evenly on SPA and incubated at 28^{o}C. Only SPA was used in this experiment as DAR75532 colonies can be easily distinguished on this medium. Plates were inspected daily and growth of DAR75532 was determined by distinguishing the colonies produced on combination plates and control plates visually.

3.2.5 Characterisation of the antagonists

The bacterial isolates characterised in the experiments described here were first retrieved from -80^{o}C and transferred to SPA. Then, they were incubated at 28^{o}C in the dark for 72 hours before being streaked for single colonies on NA for microscopic examination and characterisation.

3.2.5.1 Gram stain

A method described by Burke (1921) was used to determine the Gram stain reaction of each bacterial isolate. A loopfull of bacterial growth was picked from a 24 hour-old colony grown on NA, and smeared on a clean glass slide. Then, the slide was passed through flame to fix the bacteria. A drop of crystal violet was applied for one minute before rinsing with water, then Gram‟s iodine was added for one minute before washing the slide with water again. The smear was decolourised with Gram‟s alcohol and counterstained with saffranin for about 30 seconds. The slide was washed with water, blotted dry and examined at x1000 with oil immersion using a compound microscope (Leitz Wetzlar, Germany). Gram-positive bacteria appeared purple or bluish purple and Gram-negative bacteria were pink or red. *B. subtilis* and DAR75532, Gram-positive and Gram-negative bacteria, respectively, were used as the standards.

3.2.5.2 KOH test

A method used by Gregersen (1978) was employed in this test. A loopfull of growth was picked from a 24 hour-old colony of each bacterial isolate and emulsified separately in 10 µl of 3% KOH on a glass slide. The suspension of bacteria and KOH was then stirred with the inoculating loop for 5 to 10 seconds, then the loop was raised gently to determine if the liquid was slimy and a fine „string‟ followed the loop. A viscous, stringy suspension indicates a Gram-negative bacterium, whereas a watery suspension indicates a Gram-positive bacterium.

3.2.5.3 Growth of bacterial isolates on crystal violet agar (CVA)

Colonies from 72 hour-old cultures grown on SPA were transferred to NA agar containing 10 µg/ml crystal violet (CVA) (Barer *et al.*, 1992). The plates were again incubated in the dark at 28^{o}C for another 48 hours. DAR75532 and *B. subtilis* cultures

grown on CVA were used as the standard for the Gram-negative and Gram-positive bacteria, respectively. Gram-negative bacteria grow well on CVA while Gram-positive bacteria do not (Elliott & Des Jardin, 1999).

3.2.5.4 Identification of bacterial isolate 64161-7

Based upon the *in vitro* preliminary screening results, isolate 64161-7 was determined to inhibit growth of DAR75532. This isolate, however, was observed to produce two colony types when grown on SPA, indicating that contamination had occurred and may have affected the results during the preliminary screening. These two types were then separated by streaking for single colonies, and evaluated as single isolates or in combination against DAR75532 using the agar well diffusion technique (as described in section 2.4) with four replicates per treatment.

The isolates, referred as 64161D and 64161L were prepared on NA, incubated at 28^{o}C for 48 hours to check growth and submitted to the National Collection of Plant Pathogenic Bacteria (NCPPB), Food and Environment Research Agency, UK for identification. At the NCPPB, these two isolates were analysed by Fatty Acid Profiling and compared with commercially available TSBA6.0 and NCPPB3 libraries.

3.3 Results

3.3.1 *In vitro* screening

All eight bacterial isolates tested inhibited the growth of DAR75532. The degree of inhibition however, varied among the isolates and medium used (Figure 3.1). Generally, SPA stimulated greater antibacterial activity than NA, although isolates CBP and 71164-16 did not inhibit DAR75532 on SPA, whereas *B. subtilis* did not inhibit the growth on NA. Antagonists were considered to be efficient when they produced mean inhibition zones of 1 cm radius or more, and they were considered to be moderate when the inhibition zones produced were less than 1 cm radius. On SPA, isolates 64161-7 and PC397 efficiently inhibited growth of DAR75532, with mean radius of 1.18 and 1.12 cm, respectively. The ability of PC397 to inhibit growth of DAR75532, however, was variable among the replicate plates (Figure 3.1). The other isolates tested moderately inhibited DAR75532 on SPA, with inhibition zones of 0.92, 0.89 and 0.63 cm, respectively (Figures 3.1 and 3.2a). On NA, all isolates except *B. subtilis* moderately

inhibited DAR75532 (Figures 3.1 and 3.2b). Two-way analysis of variance indicated that the inhibition zones among the bacterial isolates and the media were significantly different at $P<0.05$. On SPA, isolate 64161-7 caused a significantly larger inhibition zone than did isolates CBP, *B. subtilis*, PC507, SUPP and 71164-16. On NA, the inhibition zone produced by 64161-7 was significantly larger than those produced by *B. subtilis*, PC397, PC507, SUPP and 71164-16.

When the surface of the agar in the inhibition zone on SPA plates with isolates 64161-7, PC397, SUPP, *B. subtilis*, PC507 and PC506 was scraped with a loop and streaked onto fresh SPA, growth of DAR75532 was evident but the colonies took 5 days to appear compared with 2 days when transferred from pure cultures. The effect of these isolates on NA was not examined.

3.3.2 Effect of volatile metabolites

On treatment plates, growth of DAR75532 was equivalent to the positive controls. There was no indication of any decline in growth of DAR75532, suggesting that there was no production of inhibitory volatile metabolites by the antagonists.

3.3.3 Inhibition of DAR75532 by CFCF

The growth of DAR75532 was not inhibited by the CFCF produced by the antagonists using either the agar well diffusion assay or the filter paper disc technique. Subsequent attempts to modify the agar well diffusion assay to detect antibiotic production by *B. subtilis* also failed to produce inhibition zones on SPA. Likewise, when different volumes of supernatant of *B. subtilis* (section 3.2.3.3) were evaluated there was no inhibition zone in the DAR75532 culture on SPA. In the experiment where DAR75532 was added into the agar before it solidified (section 3.2.3.4), CFCF of both *B. subtilis* and isolate 64161-7 also failed to inhibit the growth of DAR75532.

3.3.3.1 Assessment of antibiosis in broth culture

The effect of CFCF from isolates 64161L and PC397 on growth of DAR75532 in liquid medium is summarised in Table 3.1. When DAR75532 was inoculated into CFCF of isolate 64161L diluted 1:1 in fresh medium, only the suspension in NB+YE+G remained clear. Also in NB+YE+G, cultures of DAR75532 treated with CFCF of this

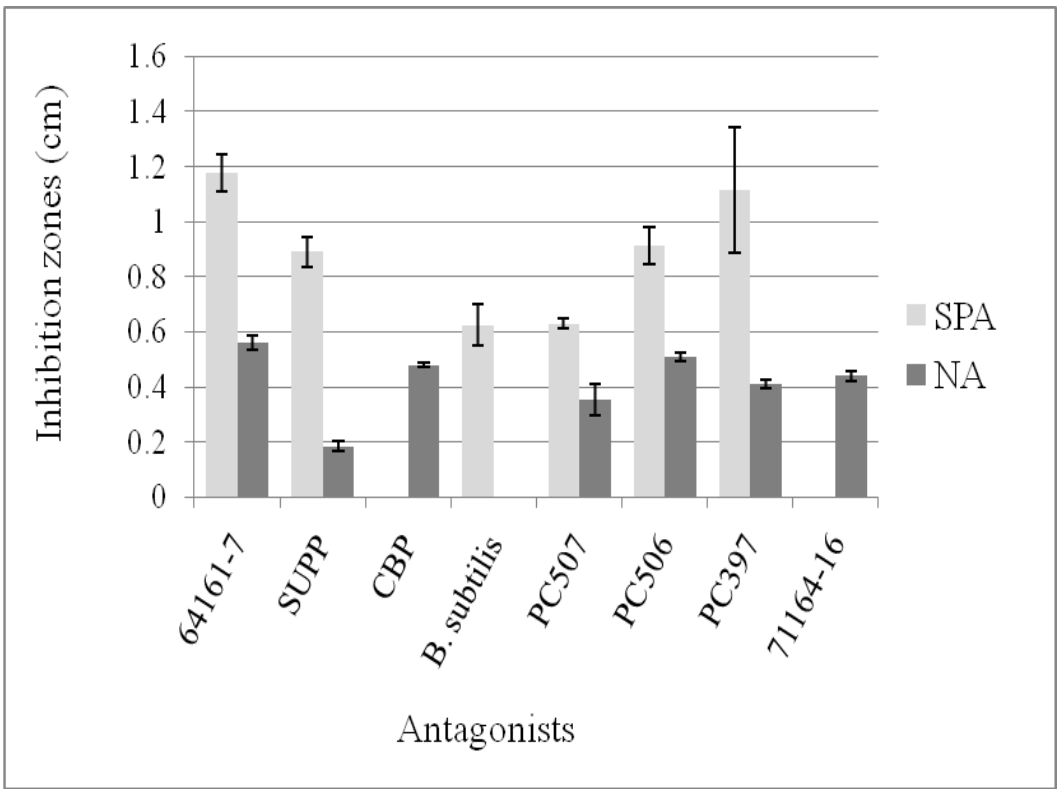
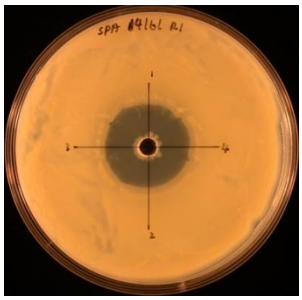
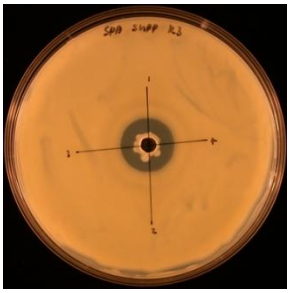
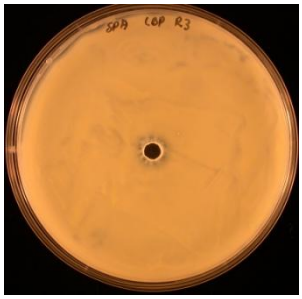
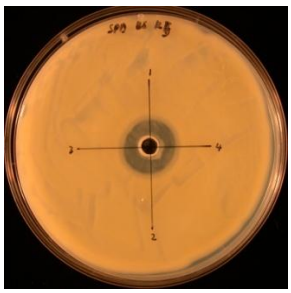


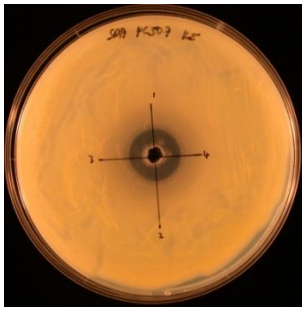
Figure 3.1: Radius of inhibition zones in the growth of DAR75532 in the presence of eight bacterial isolates when evaluated on SPA and NA using the agar diffusion technique. Each value is the mean of five replicate plates. Error bars represent 95% confidence intervals about the mean.

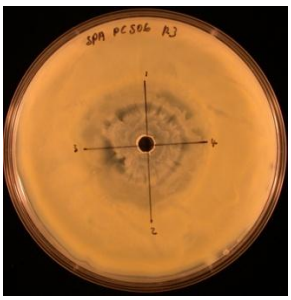

64161-7


SUPP


CBP


B. subtilis


PC507


PC506


PC397

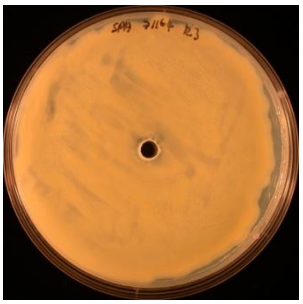
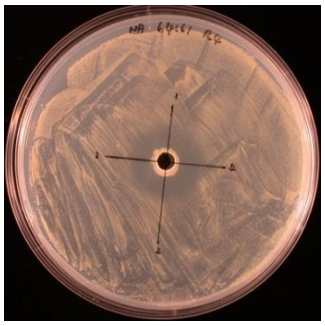
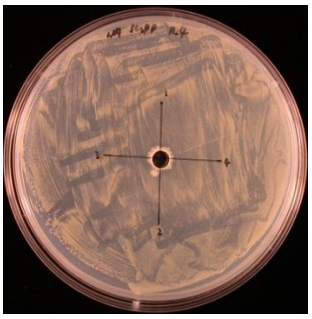

71164-16

Figure 3.2: Inhibition zones, shown as „clear" zones, produced by the potential antagonists on representative plates in the agar well diffusion assay. One hundred µl of DAR75532 containing 10^6 CFU/ml bacterial cells were spread first on SPA before a 6 mm diameter well was punched in the centre of each plate and filled with 20 µl of the antagonist. The inhibition zone was measured after 48 hours to 14 days incubation in the dark at 28^{o}C.

(a) Inhibition zones on SPA.



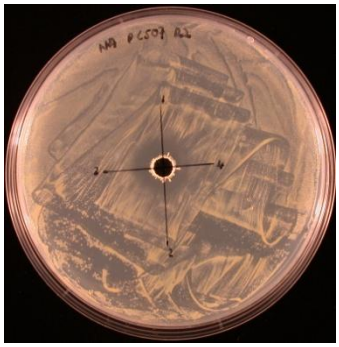
64161-7



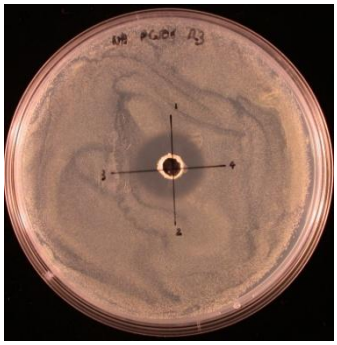
SUPP

CBP

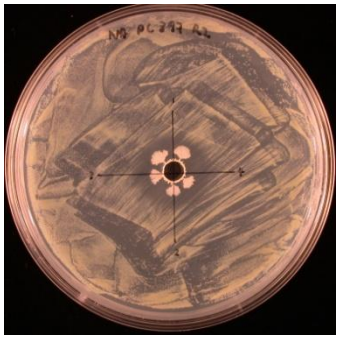
B. subtilis



PC507



PC506



PC397

71164-16

(b) Inhibition zones on NA.

isolate remained clear at all three dilutions tested. The effect of CFCF from 64161L on DAR75532 in the three media is illustrated in Figures 3.3 and 3.4. When 100 µl aliquots of clear suspensions were spread onto fresh SPA or NA+ab, DAR75532 failed to grow.

When CFCF of isolate PC397 diluted 1:1 in fresh broth was inoculated with DAR75532, suspensions in the three media remained clear (Table 3.1; Figure 3.5). In NB+YE+G, the suspensions in CFCF diluted 1:2 in fresh broth also remained clear. DAR75532 also failed to grow from aliquots transferred from the clear suspensions.

3.3.4 Competition assay

Plates spread with aliquots of antagonists 64161L, SUPP, *B. subtilis*, PC506, PC507 and PC397 combined with DAR75532, were observed to be covered with confluent growth or individual colonies of the antagonists. No colonies of DAR75532 colonies were visible on these plates. DAR75532 colonies, however, were observed on plates spread with dual cultures of DAR75532 and antagonists CBP or 71164-16.

3.3.5 Characterisation of the antagonists

Results of the Gram stain and KOH test are summarised in Table 3.2. Cells of *B. subtilis,* as the standard for Gram-positive bacteria, were bluish purple in the Gram stain and gave a watery reaction with KOH. DAR75532, as the standard for Gram-negative bacteria, produced a pink colour in the Gram stain and the cell suspension became slimy and viscous when the KOH test was conducted. In comparison with these standards, isolates SUPP, PC397, PC506 and PC507 were identified as Gram-positive and CBP, 64161L and 71164-16 as Gram-negative bacteria.

The growth of bacteria on CVA is illustrated in Figure 3.9. DAR75532 grew well on CVA, whereas *B. subtilis* grew poorly. Isolates CBP, 64161L and 71164-16 grew well on CVA, indicating that these three isolates are Gram-negative, whereas SUPP, PC397, PC506 and PC507 grew poorly or failed to grow, indicating that they are Gram-positive.

The bacterial isolates were rod-shaped and the measurements are in Table 3.2. When isolate PC397 was examined at 14 days of incubation at 28^{o}C, endospores was observed forming from the cells.

3.3.5.1 Identification of isolates 64161D and 64161L

When screened using the agar well diffusion assay, isolate 64161D produced a mean inhibition zone of 1.37 cm radius, isolate 64161L a zone of 1.62 mm radius and 1.48 mm radius when the two isolates were combined. There was no significant difference between the inhibition zones produced by these isolates or the combination of both at $P<0.05$.

Results provided by the NCPPB indicated that isolate 64161D is likely to be *Cedecea* sp., an Enterobacteriaceae. Isolate 64161L was identified as a *Pseudomonas* sp., with closest match to *P. tolaasii*, *P. fluorescens* and *P. putida*.

3.4 Discussion

In vitro antagonism tests are normally used as a first step to identify bacterial isolates that have potential to contribute to the suppression of growth of pathogenic bacteria. Here, the screening was first conducted through an agar diffusion assay in which the metabolites produced by the antagonists diffused into the solid medium, leading to inhibition of growth of DAR75532 and formation of clear zones. The radius of the inhibition zones produced by the bacterial antagonists varied among isolates. This suggested that the different isolates evaluated in this study could produce different inhibitory compounds. Similar findings were reported for bacterial isolates inhibitory to *Clavibacter michiganensis*, the cause of bacterial ring rot of potato (Gamard & De Boer, 1995). Although the amount or concentration of metabolites produced by antagonists may also affect the radius of inhibition zones (Bonev *et al*., 2008), in the present study, when the effect of different volumes of *B. subtilis* on the growth of DAR75532 was evaluated, there was no difference in the radius of inhibition zones, indicating that the amount or concentration of metabolite produced by the antagonist was unlikely to influence the outcome.

There was also variation in the size of inhibition zones produced on the two media tested, with zones generally larger on SPA than on NA. This result suggested that differences in nutrients can affect the antibacterial performance of the antagonists. Numerous studies have demonstrated that antagonistic effects *in vitro* are medium-dependent. For instance, the antagonistic activity of three strains of *P. fluorescens* and two strains of *Enterobacter* spp. on the fungus *Gibberella pulicaris* was more effective in trypticase soy broth agar (TSA) than in a semi-defined complete liquid medium

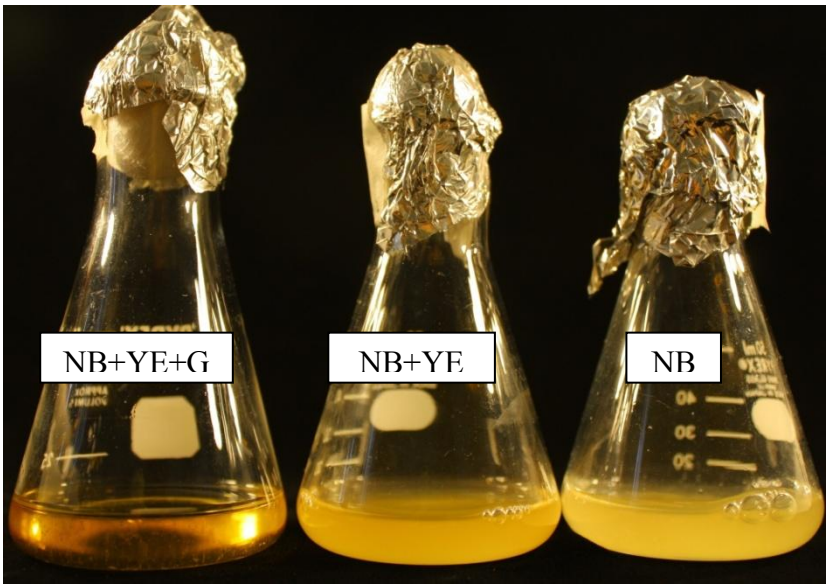
(Schisler *et al*., 2000). Also, inhibition of the fungus *Drechslera teres* by *Pseudomonas* spp. *in vitro* was stronger when evaluated on TSA and King‟s B agar than on a mineral medium agar (Borowicz & Omer, 2000).

Some of the antagonists evaluated in the present study had no obvious effect on the growth of DAR75532 on one of the two media tested. *B. subtilis,* for instance, inhibited DAR75532 only on SPA, but not on NA. Nutrients such as K_2HPO_4, $MgSO_47H_2O$, peptone and sucrose, present at a concentration of 0.05, 0.025, 0.5 and 2%, respectively, in SPA may affect the production of inhibitory compounds by *B. subtilis*. K_2HPO_4 and $MgSO_47H_2O$ increased the antagonistic activity of *B. subtilis* strain 14B against *Agrobacterium tumefaciens* when these nutrients were added to Luria Bertani medium (Hammami *et al*., 2009). Similar nutrients, plus peptone and yeast extract, in the growing medium were reported to optimise the production of bacteriocin, a secondary metabolite of *B. licheniformis* (Anthony *et al*., 2009). Likewise, bacteriocin was produced at a high level when *B. cereus* was grown in peptone broth (Bizani & Brandelli, 2004). The influence of medium composition on antibacterial or antifungal activity was also reported for other antibiotic-producing microorganisms, such as *Streptomyces* species and *Erwinia herbicola*. The greatest production of antibiotic by *S. lavendulae* was in medium supplemented with glycerol and potassium (Mellouli *et al*., 2004), whereas production of the antibiotic herbicolin A by *E. herbicola* was better in glucose-supplemented medium than with other carbon sources (Greiner & Winkelmann, 1991).

When tested following the agar well diffusion assay, the effect of all of the antagonists against DAR75532 was bacteriostatic. Indeed, when removed from the antagonist, growth of DAR75532 was restored when transferred to fresh SPA**.** Furthermore, the bacteriostatic (or bactericidal) effect of the antagonists on DAR75532 did not seem to be related to the size of inhibition zones caused by the antagonists. These effects appear to depend on the sensitivity or the threshold of the target organisms to the metabolites produced by the antagonists (Cronin *et al*., 1997; Bonev *et al.*, 2008). The recovery of DAR75532, following exposure to 64161-7, SUPP, *B. subtilis*, PC397, PC506 and PC507, however was slow, suggesting that injured DAR75532 cells required some time to repair and multiply (Rice & Bayles, 2008).

Isolates	Dilution	NB	NB+YE	NB+YE+G
64161L	1:1	+	+	-
	1:2	+	+	-
	1:4	+	+	-
PC397	1:1	-	-	-
	1:2	+	+	-
	1:4	+	+	+

Table 3.1: The effect of CFCF obtained from isolates 64161L and PC397, diluted in fresh medium, on growth of DAR75532 in broth culture (NB = nutrient broth, NB+YE = nutrient broth supplemented with 0.5% yeast extract, and NB+YE+G = nutrient broth supplemented with 0.5% yeast extract and 0.5% glucose), incubated at 27^{o}C for 4 days. + indicates that the suspension became turbid when DAR75532 was incubated with the CFCF, while - indicates that the suspension remained clear.



.

Figure 3.3: Effect of CFCF obtained from isolate 64161L on growth of DAR75532 in broth culture (from left, nutrient broth plus 0.5% yeast extract and glucose, nutrient broth plus 0.5% yeast extract, nutrient broth alone). The CFCF was diluted 1:1 with fresh medium before inoculation with DAR75532. Only the suspension on the left remained clear.

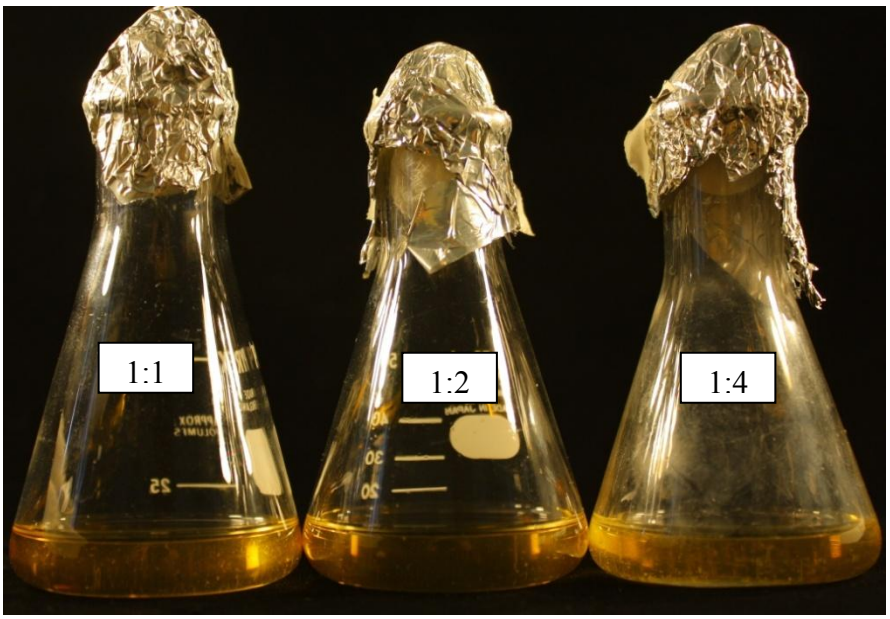


Figure 3.4: Effect of CFCF obtained from isolate 64161L on growth of DAR75532 in nutrient broth plus 0.5% yeast extract and glucose when diluted 1:1, 1:2 and 1:4 in fresh medium (from left to right). All suspensions remained clear.

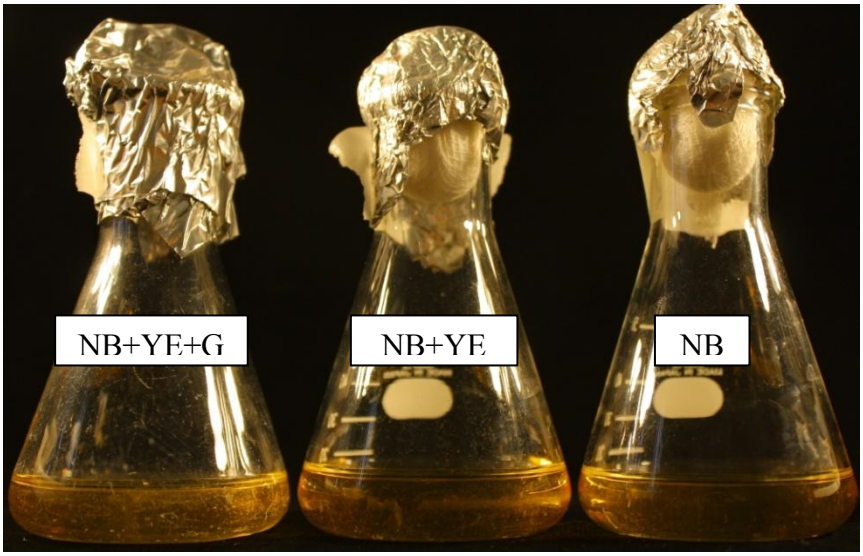
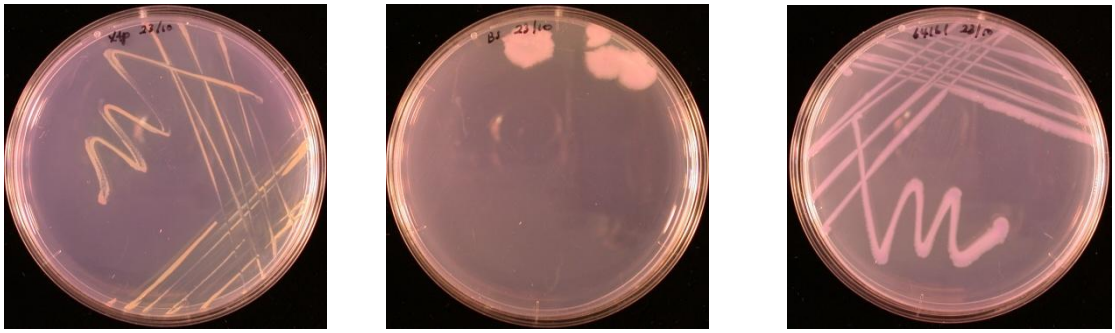


Figure 3.5: Effect of CFCF obtained from isolate PC397 on growth of DAR75532 in broth culture (from left, nutrient broth plus 0.5% yeast extract and glucose, nutrient broth plus 0.5% yeast extract, nutrient broth alone). The CFCF was diluted 1:1 with fresh medium before inoculation with DAR75532. All suspensions remained clear.

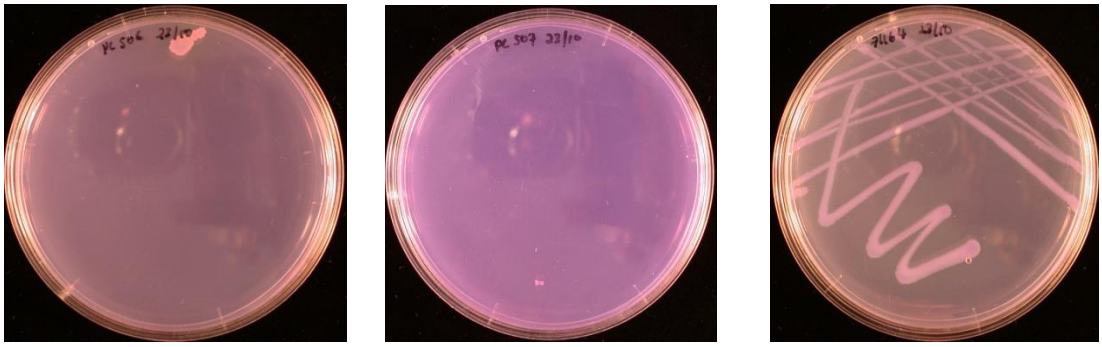
Bacterial isolates	Gram stain smear	KOH test	Measurements (µm)
B. subtilis	Bluish purple	Watery	1.2 x 3.7
DAR75532	Pink	Stringy	0.4 x 1.5
64161L	Pink	Stringy	0.4 x 1.3
71164-16	Pink	Stringy	0.4 x 1.1
SUPP	Bluish purple	Watery	1.3 x 4.0
CBP	Pink	Stringy	0.3 x 0.7
PC397	Bluish purple	Watery	0.8 x 2.7
PC506	Bluish purple	Watery	0.8 x 2.4
PC507	Bluish purple	Watery	0.5 x 1.5

Table 3.2: Summary of the Gram stain and KOH test conducted on the bacterial isolates. In the Gram stain, bluish purple colour indicates Gram-positive and a pink indicates Gram-negative. In the KOH test, slimy/viscous and stringy after treatment with 3% KOH indicates a Gram-negative bacterium, whereas a watery suspension indicates a Gram-positive bacterium.



DAR75532 *B. subtilis* 64161L

SUPP CBP PC397



PC506 PC507 71164-16

Figure 3.9: Growth of bacterial isolates on CVA. Isolate DAR75532, as the standard Gram-negative bacterium grew well on CVA and *B. subtilis*, the standard Gram-positive bacterium grew poorly. Isolates 64161L, CBP and 71164-16 grew well on CVA, while isolates SUPP, PC397, PC506 and PC507 did not.

Following the preliminary screening using the agar well diffusion assay, it was assumed that the mode of action of the bacterial antagonists was antibiosis. This was based on the evidence of diffusible substances inhibitory to DAR75532 secreted into the agar. This assumption was also supported by numerous reports on the production of antibiotics by *B. subtilis* (e.g. Asaka & Shoda, 1996; Tamehiro *et al*., 2002; Leelasuphakul *et al*., 2008). Additional experiments showed that antibacterial activity against DAR75532 was detected in concentrated culture supernatant, but not in cell-free culture filtrates. None of the attempts to evaluate the activity of CFCF on solid medium was successful in confirming that the mode of action of the bacterial antagonists against DAR75532 was through antibiotic activity. This may be due to too little antibiotic present to be effective against the target organism, or, the metabolites produced may have been inactive against DAR75532.

Another possibility is that antibiotic activity declined in solid media, or that, the technique used to detect antibiotic activity was not sufficiently sensitive. Loss of antibiotic activity either through interaction with the gelling agent of medium, aggregation or other mechanisms of inactivation was reported by Bonev *et al*. (2008). Ishimaru *et al*. (1988) also reported difficulties in detecting antibiotic activity of *E. herbicola* using the agar diffusion assay. The experiment conducted to evaluate antagonism of DAR75532 in broth culture confirmed that the agar well diffusion assay with CFCF was not sufficiently sensitive to detect antibiotic activity. Following growth of the antagonist in broth, the antibiotic in the CFCF suppressed multiplication of the pathogen in the liquid medium. Isolate 64161L seemed to produce sufficient active metabolites in medium with yeast extract and glucose to be effective when diluted 1:4. Glucose added to the medium appeared to enhance the antibiotic activity of isolate 64161L, indicating that nutrient composition influenced the production of antibiotic by this antagonist. This supports the results obtained in the preliminary screening where the antagonistic activity differed when tested in SPA and NA. It would be of interest to examine the influence of carbon source on the production of antibiotics by isolate 64161L in the future. The effect of various carbon sources on the production of antibiotic(s) by *Streptomyces antibioticus* was studied by Vilches *et al*. (1990). *S. antibioticus* produces antibiotic more effectively when grown in medium supplemented with fructose than other carbon sources, although growth was reported to be optimal in medium supplemented with glucose. Glucose and yeast extract, on the other hand, did not appear to affect the activity of PC397, although CFCF suppressed the growth of DAR75532 only at the high concentration tested.

Both isolates 64161L and PC397 produced antibiotic(s) bactericidal to DAR75532. This result is not consistent with the bacteriostatic effect found when the culture supernatant of isolate 64161L, instead of its cell-free culture filtrate, was evaluated on DAR75532 on solid medium using the agar well diffusion assay. It is possible that the bactericidal metabolites produced by this isolate were only active in liquid medium.

Another mechanism of biological control was also investigated in this study. Co-incubation of antagonists and DAR75532 in SPB suggested that competition or another form of antagonism may be involved in inhibiting DAR75532, although further work is required. This result corresponds to results obtained in the preliminary screening where isolates 64161L, SUPP, *B. subtilis*, PC506, PC507 and PC397 inhibited growth of DAR75532 on SPA. Competition is seldom reported for bacterial antagonists of plant pathogenic bacteria, but frequently reported by researchers studying biological control of postharvest disease (Mari *et al*., 1996; Bonaterra *et al*., 2003; Poppe *et al*., 2003) and interactions between bacterial antagonists and soil bacteria. For instance, *Pantoea agglomerans* suppressed the growth of *Pseudomonas savastanoi* inside the roots of olive trees by competing for space and nutrients (Marchi *et al*., 2006). Another form of competition was observed when *B. subtilis* produced a biosurfactant on *Arabidopsis* roots that protected the plant from infection by *P. syringae* (Bais *et al*., 2004).

Isolates 64161L, 71164-16 and CBP were shown to be Gram-negative while SUPP, PC397, PC506 and PC507 were Gram-positive, suggesting that the two isolates that inhibited DAR75532 most effectively *in vitro* were one of each type. Although isolate 64161-7 consisted of two colony types (designated 64161D and 64161L), when purified, they produced similar inhibition zones against DAR75532, alone or in combination, indicating that both isolates efficiently inhibited DAR75532 *in vitro*. Isolate 64161D, likely to be *Cedecea* sp., may have contaminated the original culture. Isolate 64161L, identified as *P. tolaasii*, *P. fluorescens* or *P. putida,* belonging to a genus often associated with plants, was assumed to be the original isolate and was used in subsequent experiments in this project. Based on Gram reaction, cell size, aerobic growth and production of endospores in 14 day-old culture, PC397 was considered likely to be a species of *Bacillus*.

In summary, isolates 64161L and PC397 were efficient in inhibiting the growth of DAR75532 on SPA. Antibacterial activity was more evident on SPA than NA and

the mechanisms of antagonism of these isolates appeared to be through antibiotic activity and, possibly, competition. CFCF exhibited antibacterial activity in broth culture and this seemed to offer a more sensitive detection than the agar diffusion assay. Using the broth assay, nutrient composition of the medium was found to influence the antibiosis.

CHAPTER FOUR

Colonisation of wood by *X. translucens* and antagonists

4.1 Introduction

Xtp infects the vascular system of pistachio trees. Even though the natural means of spread of the pathogen is not known, research has shown that it can become established in the plant through pruning wounds and natural wounds such as bud scars or damaged roots (Taylor *et al*., 2005; Sedgley *et al*., 2006). The bacterium appears to be confined within the xylem tissue, with minimal or no lateral spread, and has been isolated mostly from the young sapwood of the main trunk, primary and younger branches and current season growth (Facelli *et al*., 2009).

In the experiments reported in chapter 3, several bacterial isolates were identified as antagonistic to DAR75532 *in vitro*. However, inhibition of a pathogen *in vitro* does not necessarily indicate the efficacy *in planta* (Stockwell *et al*., 1998). In natural conditions, the host plant plays an important role in supporting the introduced antagonists. It is also necessary for the antagonists to be able to live and grow in the vascular tissue of the plant in order to provide long-term protection against the disease (Johnson & Stockwell, 1998). Hence, an experimental system involving the pathogen, antagonists and host plant was expected to give a more accurate assessment of potential for biological control than screening on artificial culture medium.

Efficacy of biological control of pathogens known to infect plants via mechanical or natural wounds is usually assessed by treating the wounds (Ferreira *et al*., 1991). For example, suspensions of *Eutypa lata* (pathogen) and *Bacillus subtilis* or *Erwinia herbicola* (antagonists) were applied to cuts in the wood of grapevines, and the efficacy of the antagonist was evaluated by observing the growth of pathogen in the treated wood (Schmidt *et al.*, 2001a). In pistachio, application of *Xtp* to the wounds mimics what would happen when pistachio trees are pruned with contaminated tools during routine orchard management operations (Taylor *et al*., 2005; Sedgley *et al*., 2006). Therefore, an approach similar to that reported by Ferreira *et al*. (1991) and Schmidt *et al.* (2001a) could be useful for studies of biological control of *Xtp* in pistachio. In this chapter, studies of the ability of the bacterial antagonists to colonise

pistachio wood and reduce colonisation by *Xtp* are reported. Experiments undertaken to ensure that the pathogen became established in the wood tissue are also reported.

4.2 Materials and Methods

4.2.1 Preliminary assessment of colonisation of wood by DAR75532 and antagonists

A method developed by John *et al*. (2004) with some modifications was used initially to assess the ability of the antagonists to reduce colonisation of detached pistachio twigs by the pathogen. All antagonists evaluated in the experiments described in chapter 3 were used in this experiment. Isolate DAR75532 of the pathogen was used to maintain consistency between experiments. Pieces of pistachio twigs (50 mm long and 10 to 15 mm diameter) were collected from healthy, un-inoculated grafted trees (cultivar Sirora, aged 3 years), growing in the shade-house at Waite Campus, University of Adelaide. After removing the bark using a scalpel, the twigs were placed upright into McCartney bottles, with the proximal part (i.e. which was closest to the point of attachment) immersed in 3 ml of SDW, loosely capped and autoclaved at 121^{o}C for 20 minutes.

Suspension of DAR75532 containing 10^6 CFU/ml was prepared using SDW as described in section 2.2. Likewise, suspensions of the antagonists were prepared (section 2.2) but the concentration was not determined in this preliminary assessment. When the twigs had cooled down to room temperature, 100 µl of suspension of DAR75532 was applied aseptically to the upper cut surface (distal end, i.e. which was furthest from the point of attachment) of each twig and the lid of the bottle was replaced loosely. About half an hour later, the same wound surface was treated with 100 µl of antagonist suspension. Each antagonist was evaluated separately. The experiment was replicated five times and excised twigs treated either with pathogen only or antagonist only served as controls. The lids were placed on the bottles and the wood segments were then incubated in the dark at 28^{o}C for 7 days before assessing colonisation of the twig by DAR75532 or the bacterial antagonists, as described below.

4.2.2 Assessment of the colonisation of wood by bacteria

Colonisation of the pistachio wood segments was assessed by re-isolating both the antagonist(s) and the pathogen as follows (John *et al*., 2005). Each wood segment was surface sterilised with 95% ethanol and flamed before cutting discs, about 2 mm thick, 1, 2 and 3 cm from the inoculation site. Three to 5 chips were cut from each section and transferred to SPA. Plates were then incubated at 28^{o}C in the dark. Plates were inspected after 48 hours of incubation and then daily for another 14 days. The wood chips were considered to be colonised if any colonies of DAR75532 or antagonists grew from them onto the agar. For wood chips treated with both pathogen and antagonist, the pathogen and antagonists were monitored by transferring the culture grown from the chips onto fresh SPA and colonies of each were distinguished visually.

4.2.3 Refinement of methods for colonisation of wood by DAR75532

Results obtained from the experiment described in section 4.2.1 indicated some inconsistency in the recovery of DAR75532 from wood segments inoculated with the pathogen alone. Therefore, a method to ensure that DAR75532 was effectively introduced into the wood segments needed to be designed. Several modifications were made to the concentration of DAR75532 applied, the medium used for preparing suspension of DAR75532 and the duration of incubation of DAR75532 in the wood. The method for preparing the wood segments before applying the pathogen was also adjusted. Experiments to evaluate each factor were conducted separately and each treatment was replicated at least four times.

To determine an appropriate concentration of *Xtp* for inoculation purpose, DAR75532 at concentrations of 10^{7} to 10^{10} CFU/ml was evaluated, applied at a standard volume of 100 µl with SDW for controls (experiment 1). A separate experiment (experiment 2) was conducted to test the effect of nutrient supplement on the ability of the pathogen to colonise pistachio twigs. Here, DAR75532 was suspended in SPB instead of SDW, and concentrations of 10^{8} and 10^{10} CFU/ml used. Suspensions of DAR75532 prepared in SDW were used for comparison. The method of inoculation described in section 4.2.1 was used in both the experiments.

To determine if the method of preparing the wood segments affected the recovery of the pathogen, wood segments were inoculated after autoclaving or without

being autoclaved beforehand (experiment 3). The bark was left intact or removed, the twigs were then surface sterilised by dipping in ethanol and flaming, then were placed upright in 3 ml SDW in McCartney bottles. DAR75532 at 10^6 or 10^7 CFU/ml prepared in SDW, or a loopfull of DAR75532 plus slime scraped directly from a 72-hour-old culture grown on SPA was applied on the cut surface. The wood segments were then incubated at 28^{o}C for 7 days before re-isolating the pathogen on SPA, as described in section 4.2.2.

The effect of incubation period of DAR75532 on the recovery of the pathogen on SPA was also examined (experiment 4). Twigs with the bark removed were placed upright in 3 ml SDW and autoclaved. The cut surface of the wood was treated with 100 µl of DAR75532 at 10^8 CFU/ml in SPB or one loopfull of bacteria plus slime, then incubated in the dark at 28^{o}C before re-isolating the pathogen onto SPA (section 4.2.2). Sterile non-absorbent cotton wool was used to close the McCartney bottles to improve gas exchange. The incubation periods evaluated were 1, 2, 3, 4 and 5 days.

4.2.4 Development of vacuum infiltration method for inoculation of pistachio wood

Application of DAR75532 to the cut surface of pistachio twigs failed to produce consistent growth of the pathogen when the wood was dissected and cultured on SPA. An alternative method of inoculation, developed in collaboration with Professor Giora Kritzman, was used as follows. Suspensions of DAR75532 and the bacterial antagonists were infiltrated into the excised pistachio twigs under vacuum using the apparatus shown in Figure 4.1. In this experiment, only antagonists 64161L and PC397 were evaluated, based on their effectiveness in inhibiting the growth of DAR75532 on SPA (section 3.3.1), due to time constraints.

Suspensions of DAR75532 and the antagonists were prepared from 72-hour old cultures grown on SPA. The cultures were flooded with 10 ml of 0.085% sodium chloride solution (sterile saline) and the cells were gently suspended using a sterile glass spreader. The suspensions were then transferred to sterile 10 ml screw capped centrifuge tubes, and 100 µl from each suspension were spread onto SPA, for enumeration of CFU. Pistachio twigs, obtained from healthy trees growing in the shade-house as described in section 4.2.1, were dipped in ethanol and flamed before attaching the distal end of the twig onto a plastic tube of 10 mm diameter. The twig was attached firmly to the tube using Parafilm, so that it became airtight to improve suction. The tube

was attached to a vacuum pump (HETOSUC®, Scandinavia). The excised twigs were then infiltrated with pathogen only, antagonist only, water only (controls), or a combination of DAR75532 plus antagonist in a single suspension for about one minute. Infiltration of suspension for longer than this would result in bacteria being carried right through the wood and might contaminate the tube. The mixed suspension was prepared immediately before vacuum infiltration and each treatment was replicated five times.

After infiltration, twigs were kept in a sterile plastic Petri dish of 90 mm diameter, five twigs (each treatment) per plate, sealed with Parafilm, and incubated at 28°C in the dark. After 10 days, the middle 1 cm section of each twig was cut, weighed aseptically and sliced into four pieces before immersing the four pieces in a tube containing 9 ml of sterile saline. The tubes were then placed on an orbital shaker at 3000 rpm and incubated at 28°C for an hour. The suspensions were then serially diluted to 10^{-4} in sterile saline. Aliquots of 100 µl were taken from the diluted suspension and spread evenly onto SPA and NA supplemented with cephalexin, ampicillin and gentomicin (NA+ab, as described in Appendix 1), one plate of each medium per tube. The plates were incubated again at 28°C in the dark. Colonies of DAR75532, 64161L and PC397 produced on the two media were distinguished visually and enumerated. The weight of the wood chips and number of microbial colonies were used to estimate the number of viable cells per gram of wood.

This experiment was repeated by Professor Giora Kritzman, and colonisation of the proximal end of the infiltrated wood, about 1 cm from the inoculation point, and distal end about 4.5 cm from inoculation point was also assessed. The mean values for CFU/g of wood were then tested for significant differences by analysis of variance, and the least significant difference test was used to compare treatment means at $P<0.05$.

4.3 Results

4.3.1 Preliminary assessment of colonisation of wood by DAR75532 and antagonists

The antagonists were recovered on all replicate plates from all twigs at 1, 2 and 3 cm from the inoculation site in the negative (antagonist alone) controls. However, DAR75532 was not recovered from the positive controls where the pathogen only was

applied. Colonies of the antagonists were also recovered from all twigs treated with the combination of pathogen and antagonists (Figure 4.2).

4.3.2 Refinement of methods for colonisation of wood by DAR75532

The results obtained from the experiments are summarised in Table 4.1. When inoculum concentrations of 10^7 to 10^{10} CFU/ml of DAR75532 in SDW were evaluated, none of the resulting plates showed growth of the pathogen (experiment 1 in table 4.1). When DAR75532 inoculum was prepared in SPB, DAR75532 was recovered from twigs inoculated with both 10^8 and 10^{10} CFU/ml (experiment 2 in table 4.1). However, of seven replicate plates, only two and one replicate plates had colonies of DAR75532, recovered from twigs treated with 10^8 and 10^{10} CFU/ml, respectively. Of these plates, only one replicate had evidence of colonisation of DAR75532 at 1, 2 and 3 cm from the inoculation site. Where DAR75532 was applied as a suspension in SDW, no DAR75532 colonies were observed on any of the plates.

DAR75532 was recovered from both autoclaved and non-autoclaved twigs inoculated with bacterial slime but recovery was not consistent on the replicate plates (experiment 3 in table 4.1). Of four replicates of autoclaved wood, only two had growth of DAR75532 whereas three replicates of non-autoclaved wood yielded DAR75532. Of these replicates, only one non-autoclaved twig was colonised by DAR75532 at all the three positions assessed. DAR75532 was also recovered from autoclaved twigs inoculated with 10^8 CFU/ml when harvested after 3 and 5 days of incubation (experiment 4 in table 4.1). However, not all the replicate plates from these treatments produced growth of DAR75532. Only three of four replicates (Figure 4.3) and two of four replicates from 3 and 5 days of incubation, respectively, had the evidence of DAR75532 colonies on SPA plates. Colonisation by DAR75532 up to 3 cm from the inoculation site was observed on one replicate plate when the wood segment had been incubated for 5 days.

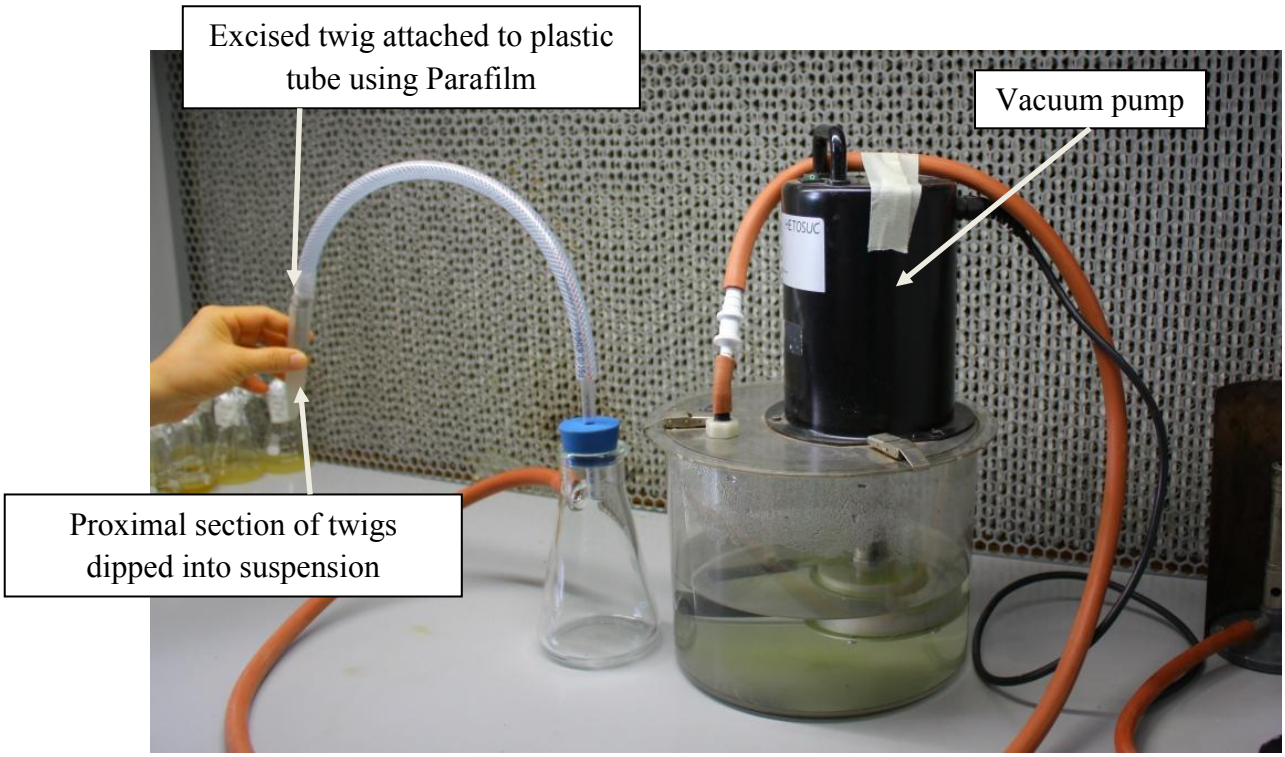


Figure 4.1: The apparatus used to infiltrate excised pistachio twigs with DAR75532, antagonist, or DAR75532 plus antagonist. The excised twig was surface sterilised with 95% ethanol and flamed before attaching the distal end to a plastic tube of 10 mm diameter. The proximal end was dipped into suspension of DAR75532, antagonist or DAR75532 plus antagonist and the vacuum applied for about one minute.

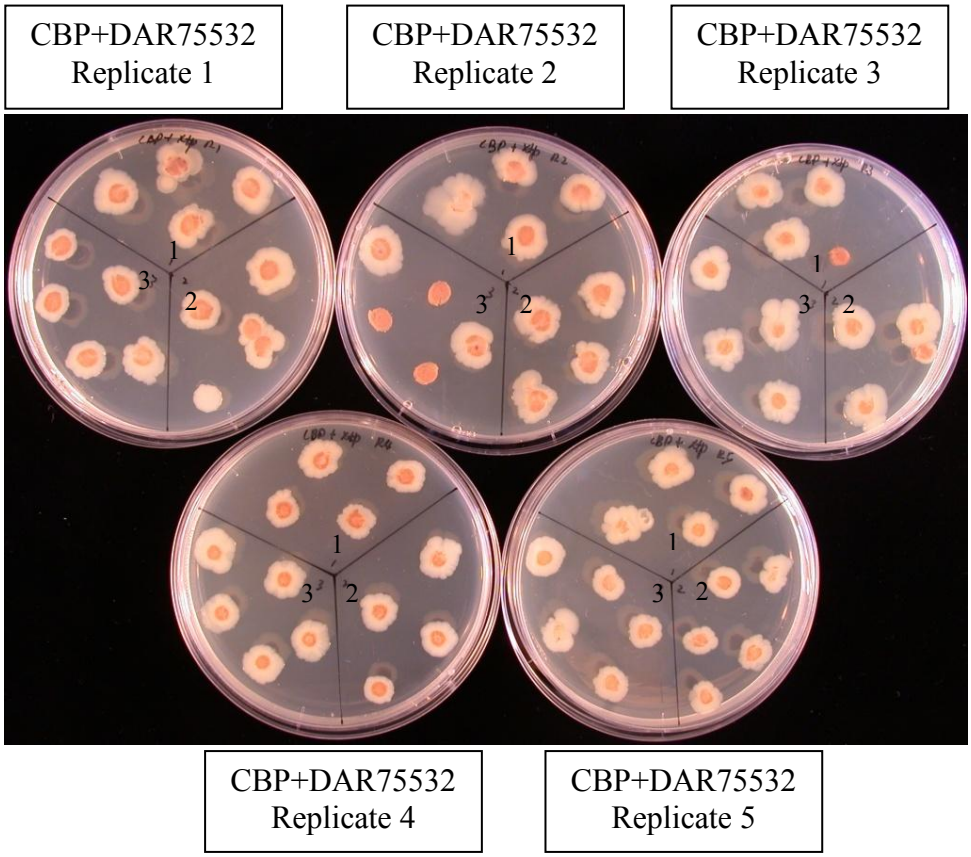


Figure 4.2: Example of antagonist recovered from twigs inoculated with DAR75532 plus isolate CBP following incubation of wood chips on SPA. DAR75532 was prepared in SDW and inoculated at 10^6 CFU/ml onto the cut surface of autoclaved pistachio twigs, then suspension of the antagonist CBP was applied to the same wound. After 7 days of incubation, the wood segment was surface sterilised before cutting discs, measured 1, 2 and 3 cm from the inoculation site, into 3 to 5 chips per disc. Each plate was divided into three sections (1, 2 and 3) corresponding to the 1, 2 and 3 cm sections of the twigs, respectively. Plates were incubated at 28^{o}C in the dark. Colonies of pathogen and antagonist were monitored after 48 hours and daily for another 14 days.

E	Treatment	Reps	Replicate (R) twigs and percentage of wood chips colonised by DAR75532		
			1 cm	2 cm	3 cm
1	**10^7 CFU/ml in SDW**	4	0	0	0
	10^8 CFU/ml in SDW	4	0	0	0
	10^9 CFU/ml in SDW	4	0	0	0
	10^{10} CFU/ml in SDW	4	0	0	0
	SDW (control)	4	0	0	0
2	**10^8 CFU/ml in SPB**	7	R2(50) R5(50)	R5(100)	R5(100)
	10^8 CFU/ml in SDW	7	0	0	0
	10^{10} CFU/ml in SPB	7	R4(40)	0	R4(20)
	10^{10} CFU/ml in SDW	7	0	0	0
3	**10^6 CFU/ml in SDW to autoclaved wood**	4	0	0	0
	10^6 CFU/ml in SDW to non-autoclaved wood	4	0	0	0
	10^7 CFU/ml in SDW to autoclaved wood	4	0	0	0
	10^7 CFU/ml in SDW to non-autoclaved wood	4	0	0	0
	DAR75532 + slime to autoclaved wood	4	R1(100) R4(100)	R4(50)	R1(20)
	DAR75532 + slime to non-autoclaved wood	4	R2(50) R3(100) R4(20)	R2(20) R3(100)	R3(100)
4	**10^8 CFU/ml in SPB (1 day)**	4	0	0	0
	10^8 CFU/ml in SPB (2 days)	4	0	0	0
	10^8 CFU/ml in SPB (3 days)	4	R1(100) R2(25) R3(100)	R1(100) R3(25)	0
	10^8 CFU/ml in SPB (4 days)	4	0	0	0
	10^8 CFU/ml in SPB (5 days)	4	R3(100) R4(20)	R3(20) R4(50)	R3(20)
	DAR75532 + slime (1 day)	4	0	0	0
	DAR75532 + slime (2 days)	4	0	0	0
	DAR75532 + slime (3 days)	4	0	0	0
	DAR75532 + slime (4 days)	4	0	0	0
	DAR75532 + slime (5 days)	4	0	0	0

Table 4.1: Summary of results obtained in four experiments (E1-4) to refine methods for inoculation of pistachio twigs by DAR75532. Four factors were investigated; 1: different inoculum concentrations prepared in SDW, 2: DAR75532 suspensions prepared in SPB and SDW, 3: inoculation of autoclaved and non-autoclaved twigs, and 4: incubation period of DAR75532, given in brackets. After inoculation with DAR75532, wood segments were incubated for 7 days (except in experiment 4) at 28^{o}C in the dark, then wood segments were cut at 1, 2 and 3 cm from the inoculation site and transferred to SPA. Plates were then incubated at 28^{o}C in the dark. After 48 hours, plates were inspected daily for 14 days for growth of DAR75532. Only replicates with a positive result are presented, with the percentage of wood chips colonised by DAR75532 in the brackets.

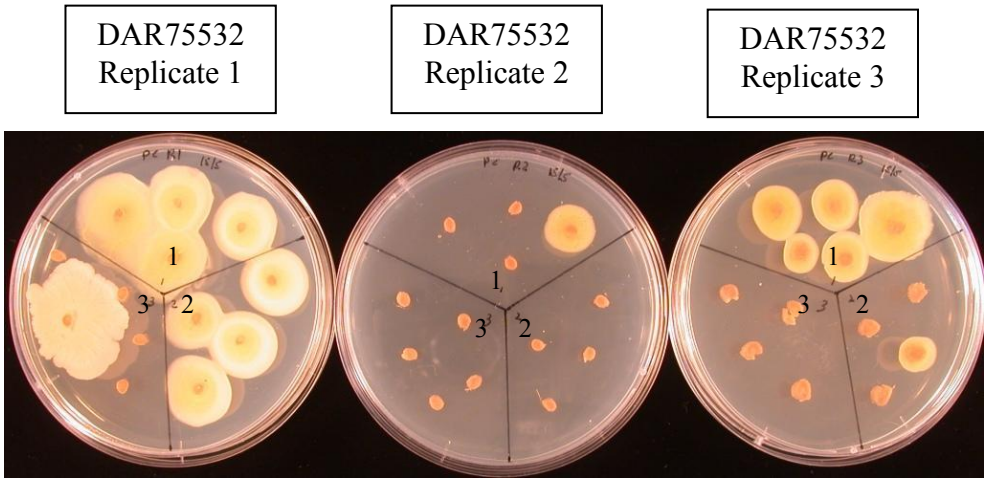


Figure 4.3: Example of the inconsistent results obtained from the positive (DAR75532 alone) controls. Suspension of DAR75532 was prepared in SPB and inoculated at 10^8 CFU/ml onto the cut surface of autoclaved twigs. After 3 days of incubation at 28^{o}C in the dark, the wood segment was surface sterilised before cutting discs, measured 1, 2 and 3 cm from the inoculation site, into 3 to 5 chips each cm and plated onto SPA. On Replicate 1, all discs at 1 and 2 cm from inoculation site were colonised by DAR75532 and at 3 cm, one disc was contaminated with wood-inhabiting bacteria. On Replicate 2, of four discs at 1 cm from inoculation site, only one yielded DAR75532. On Replicate 3, all discs at 1 cm and one disc at 2 cm from inoculation site were colonised by DAR75532.

4.3.3 Development of vacuum infiltration method for inoculation of pistachio wood

The results from this experiment are summarised in Table 4.2. The initial inoculum concentrations determined through the plate-counting technique were 6.1 x 10^8 CFU/ml for DAR75532, 2.5 x 10^8 CFU/ml for 64161L and 6.0 x 10^9 CFU/ml for PC397. For the positive controls where only DAR75532 was applied to the twigs, the pathogen was recovered from all replicate plates of SPA and NA+ab. The growth of DAR75532 on NA+ab was more obvious and readily detected, whereas on SPA, indigenous wood-inhabiting bacteria grew on the plates and obscured colonies of DAR75532. Therefore, only colonies of *Xtp* on NA+ab were counted and the average recovery was 6.1 x 10^6 CFU/g. For the negative controls, where either 64161L or PC397 was applied to the twigs, the average recovery of the antagonists was 3.4 x 10^7 CFU/g and 5.9 x 10^7 CFU/g, respectively. The colonies were counted only on SPA as neither antagonist grew on NA+ab.

The recovery of DAR75532 from twigs infiltrated with pathogen plus either one of antagonist on NA+ab was significantly less ($P<0.05$) than that from the positive controls. For twigs infiltrated with DAR75532 plus isolate 64161L, the number of DAR75532 colonies recovered was 5.1 x 10^6 CFU/g, whereas on SPA, both DAR75532 and 64161L were recovered although indigenous wood-inhabiting bacteria also grew. From twigs treated with DAR75532 plus PC397, only PC397 was recovered on SPA with the average recovery of 1.0 x 10^7 CFU/g. Neither pathogen nor antagonist was recovered on NA+ab. The population density of pathogen recovered from twigs infiltrated with DAR75532 plus 64161L was significantly larger than that from twigs treated with DAR75532 plus PC357 ($P<0.05$).

Results obtained from the experiment conducted by Professor Giora Kritzman are presented in Figure 4.4. DAR75532 was recovered consistently from the positive controls, as were the antagonists from the negative controls (not shown). Two-way analysis of variance indicated that the population density of DAR75532 recovered from the positive controls was significantly higher (at $P<0.05$) than that recovered from twigs treated with pathogen plus 64161L or PC397. However, there was no significant difference between the number of DAR75532 colonies recovered from wood treated with DAR75532 plus 64161L and DAR75532 plus PC357. The population of pathogen recovered from the treatments and positive controls at 1 cm from the inoculation site was significantly larger than those recovered at 2.5 and 4.5 cm from the inoculation site.

	Initial inoculum concentration (CFU/ml)	Recovery of DAR75532 or antagonists (CFU/g)			
		SPA		NA + ab	
		DAR75532	Antagonist	DAR75532	Antagonist
DAR75532 (positive control)	6.1×10^8	✧	NT	6.1×10^6	NT
64161L (negative control)	2.5×10^8	NT	3.4×10^7	NT	0
PC397 (negative control)	6.0×10^9	NT	5.9×10^7	NT	0
64161L + DAR75532	$(2.5 \times 10^8) + (6.1 \times 10^8)$	✧	✧	5.1×10^6 *	0
PC397 + DAR75532	$(6.0 \times 10^9) + (6.1 \times 10^8)$	0	1.0×10^7	0 *	0
				LSD=5.3×10^5	

Table 4.2: Recovery of bacteria following vacuum infiltration into detached pistachio twigs. DAR75532, antagonists 64161L and PC397, or DAR75532 plus one of the antagonist were vacuum-infiltrated into non-autoclaved excised pistachio twigs (5 cm in length), five replicates per treatment, before incubating the twigs for 10 days. The pathogen and antagonists were recovered from the middle 1 cm of the wood, following soaking in 0.085% sodium chloride solution and plating suspensions on SPA and NA+ab. ✧ indicates that the pathogen and the antagonist were recovered on the medium, but other wood-inhabiting bacteria grew as well. NT (not tested) indicates that observation of the particular bacterium was not applicable. All data represent the means of five replications. Asterisk (*) indicates mean was significantly different from positive control, LSD at $P<0.05$.

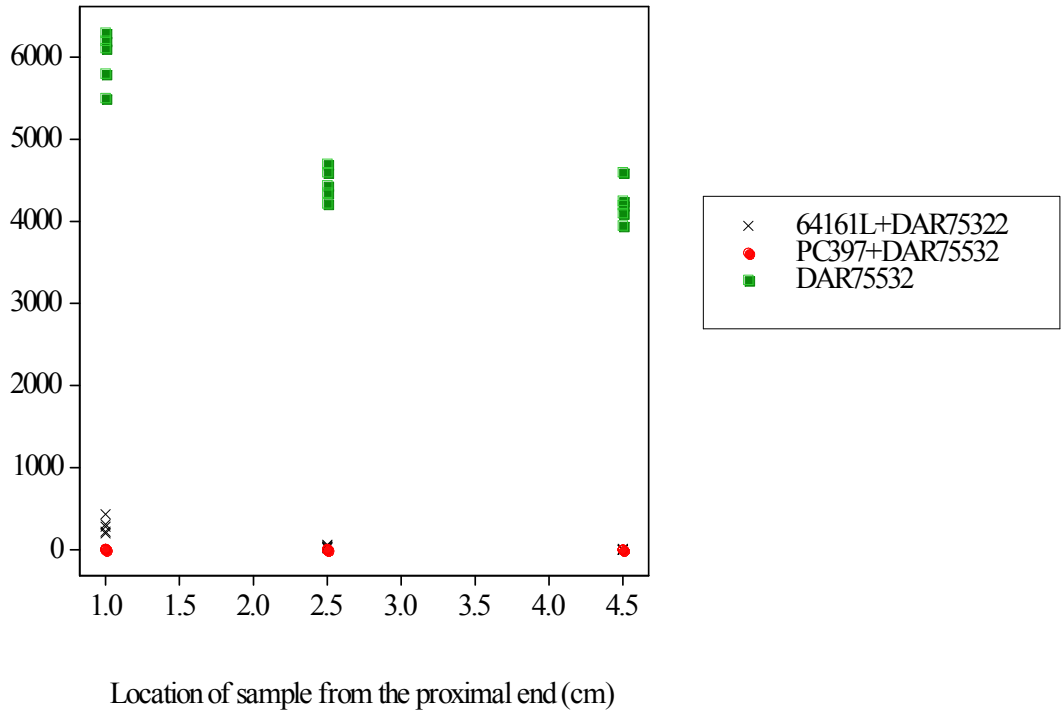


Figure 4.4: Pattern of population of DAR75532 recovered from the excised twigs infiltrated with DAR75532 (positive control), DAR75532 plus PC397 and DAR75532 plus 64161L, at 1, 2.5 and 4.5 cm from the inoculation point (proximal end) after 10 days of incubation at 28^{o}C. Each data point represents one replicate with LSD = 113.5 at $P<0.05$.

The population declined as sampling was taken further from the inoculation site, however, there was no significant difference between the population density of DAR75532 recovered at 2.5 and 4.5 cm ($P<0.05$). When the twigs were cut in half longitudinally, staining was evident in wood infiltrated with DAR75532 alone but was not observed in twigs infiltrated with SDW (control) or DAR75532 plus PC397 (Figure 4.5). Twigs infiltrated with DAR75532 plus 64161L were stained only at the proximal end.

4.4 Discussion

Although the bacterial antagonists could be recovered from the inoculated twigs in the preliminary experiment, DAR75532 could not, suggesting that the method based on John *et al.* (2004) was not suitable to examine the colonisation of wood by this pathogen. In these conditions, the effect of the antagonist on the colonisation of DAR75532 in the wood could not be compared adequately with the positive controls. Therefore, considerable time and effort was devoted to developing a method suitable for delivering pathogen inoculum into the excised pistachio twigs. The survival and the establishment of the pathogen or antagonists in the inoculated wood needed to be examined as well.

As DAR75532 was not recovered from excised twigs inoculated on the cut surface with 100 µl of a suspension with less than 10^8 CFU/ml or when no additional nutrients were provided, this suggested that high concentrations of inoculum and nutrients may be required to artificially infect wood with DAR75532 *in vitro*. The performance of bacteria as biological agents may be enhanced by modifying the formulation, for example, *B. subtilis* applied in $MgSO_4$ solution supplemented with peptone and methylcellulose inhibited colonisation of autoclaved grape wood by *E. lata* more effectively than when applied in the $MgSO_4$ solution alone (Schmidt *et al*., 2001b).

Autoclaving or heat treatment of wood has been shown to affect colonisation of wood by microorganisms (Shortle & Cowling, 1978). Autoclaved wood has frequently been used in evaluating the efficiency of biological control agents (Schmidt *et al*., 2001a; Velmurugan *et al*., 2009). For example, the inhibition of *E. lata* by 391 bacterial isolates tested was greater in autoclaved excised grape canes than in non-autoclaved canes (Munkvold & Marois, 1993).

Figure 4.5: Representative twigs infiltrated from the proximal end with (from left) SDW (control), DAR75532, DAR75532 plus 64161L and DAR75532 plus PC397. Twigs were incubated at 28^{o}C for 10 days then cut in half longitudinally and staining examined.

Sterilisation of pistachio twigs was used initially to prevent competition from indigenous wood-inhabiting bacteria. However, there was no obvious difference between autoclaved or non-autoclaved wood in terms of colonisation by DAR75532. Non-autoclaved pistachio twigs were used by Sedgley *et al*. (2006) to study the effect of quaternary ammonium bactericide on *Xtp*. The pathogen was recovered from the top 1 cm from the inoculation site in the positive controls from wood inoculated with low inoculum concentration of 10^4 CFU/ml (Sedgley *et al*. 2006), contradicting the finding in the present study. The experiment designed to investigate the time required for DAR75532 to colonise the twigs was inconclusive.

In the above-mentioned experiments, the pathogen and antagonists were introduced into the wood by applying suspensions onto the distal surface of the excised twigs. Although DAR75532 was sometimes recovered from the positive controls, variation was observed among replicate plates and there was no clear pattern in the length of twig colonised by the pathogen. Therefore, measuring the extent of colonisation by cutting the twigs into segments from the inoculation site downwards and plating them directly onto SPA seemed insufficient to evaluate colonisation and antagonism. Furthermore, this method could not be used to quantify the density of pathogen or antagonist cells recovered from the inoculated wood. Sedgley *et al*. (2006) used a slightly different technique for evaluating recovery of the pathogen from inoculated excised pistachio wood. Instead of directly plating the inoculated twigs onto selective medium, the wood was soaked in SDW overnight, and the resulting suspension was then spread on SPA supplemented with Benlate. In addition, Sedgley *et al*. (2006) showed that the movement of *Xtp* in living, young pistachio trees, detected by plating and the production of staining, was more than 50 cm from the inoculation point after 1 week. In comparison, *P. syringae*, the causal organism of canker, moved 3 cm from the inoculation site in the stems of young pear trees in 20 days (Whitesides & Spotts, 1991). This indicates that *Xtp* may be able to colonise woody tissues of living trees rapidly compared with some other bacterial pathogens of woody plants. Evidently, the inoculation of excised twigs *in vitro* did not reflect the capacity of the bacterium to infect and colonise pistachio trees.

Vacuum infiltration appeared to improve the reliability of inoculation of the twigs, even though the two methods of inoculation were not compared directly in the present study. This is supported by a study published by Hélias *et al*. (2000), in which vacuum infiltration of *E. carotovora*, the potato soft-rot pathogen, into seed tubers was

more efficient in introducing the pathogen than inoculating via wounds made by shaking tubers in infested sand. Plants from tubers infiltrated with pathogen showed earlier and more severe symptoms of disease than plants from tubers inoculated on wounds. However, vacuum infiltration, especially from the proximal end of the twig, will not necessarily reflect the natural means of colonisation of pistachio trees by *X. translucens* pv. *pistaciae*, hence experiments involving inoculation of living trees are required.

The vacuum infiltration method allowed DAR75532 to be recovered consistently from the positive controls, both on SPA and NA+ab. However, only colonies grown on NA+ab were enumerated. SPA was heavily contaminated with other wood-inhabiting bacteria, but it has the advantage of facilitating the recognition of DAR75532, and therefore was used to confirm colonies produced on NA+ab as DAR75532 following subculture. The recovery of DAR75532 using this method could be improved in future studies by using SPA plus antibiotics to inhibit indigenous wood-inhabiting bacteria.

After 10 days of incubation, the population of DAR75532 recovered from the positive controls decreased from 6.1 x 10^8 CFU/ml to 6.1 x 10^6 CFU/g wood and the populations of the antagonists also decreased. However, it is possible that not all viable cells of DAR75532 were recovered due to the nature of the wood, so there may be limitations in comparing CFU of DAR75532 recovered from wood with CFU applied in the inoculum suspension. In future experiments, the amount of suspension infiltrated into the wood should be quantified and recovery of bacteria from representative twigs should be assessed immediately after inoculation to determine the population per gram of wood at time zero. Nevertheless, Stockwell *et al*. (1998) reported that the detectable population of *P. fluorescens*, an antagonist of the fire blight pathogen, in blossoms of apple and pear was between 10^3 to 10^6 CFU per blossom after 2 to 8 days of incubation, a decline from the inoculum concentration of 10^8 CFU/ml used in the spray application. In contrast, the population density of *P. corrugata*, an endophyte with potential to control Fusarium wilt, increased in the stem tissue of inoculated cotton plants (Chen *et al*., 1995); following inoculation with 10^3 CFU/ml the population of *P. corrugata* increased to log 6.25 CFU/g stem tissue 3 days after inoculation. In addition, the recovery of *Xylella fastidiosa*, the causal agent of Pierce‟s disease in grapevine, 113 days after inoculation was influenced by the grape genotype (Fritschi *et al*., 2007). When inoculated with 6 x 10^8 CFU/ml of *X. fastidiosa*, the recovery of the pathogen

from wood of resistant genotypes was less than 2.1 x 10^6 cells/g tissue but was 1 x 10^8 cells/g in the less resistant genotypes tested. The influence of different genotype of pistachio on the recovery rate of DAR75532 or the antagonists was not studied as only one scion cultivar is grown commercially in Australia and the main purpose of the experiment was to obtain consistent results in the controls.

The lack of obvious increase in the population of DAR75532 and the antagonists in the inoculated pistachio twigs suggested that neither grew well in the wood in the conditions tested. This could be due to the orientation of twigs during the infiltration process. Suspension of DAR75532 or antagonist was infiltrated from the proximal to the distal part of the twig, following the normal transpiration flow of water in the tree (Davis *et al.*, 1980). As the pathogen appears to enter via pruning wounds and biological control most probably would be applied to living trees via pruning wounds, the movement of the bacteria would then be from distal to proximal parts of the tree. Therefore, it would be appropriate to repeat the experiment with infiltration from the distal to the proximal part of the twig. Another reason could be that the experiment was done on excised twigs *in vitro* rather than on the living tree. As noted above, Sedgley *et al*. (2006) detected *Xtp* 50 cm from the inoculation point in the living pistachio trees and the increase in the population of bacteria after inoculation reported by Chen *et al*. (1995) was observed on live plants, and not on detached plant parts as was done in the current study. Plant defence mechanisms might also affect the population of bacteria recovered after inoculation. It is well-documented that plants produce antimicrobial compounds in response to bacterial invasion. Phytoalexins are produced and the activity of oxidase is increased when the plant is infected by bacteria (Smith, 1996).

For all twigs infiltrated with DAR75532 plus isolate 64161L, DAR75532 was recovered on all replicate plates, however, the average population recovered was less than in the positive controls, indicating that isolate 64161L reduced colonisation by or survival of DAR75532 to a certain degree. DAR75532 was not recovered from twigs infiltrated with DAR75532 plus PC397 in the first infiltration experiment, which indicated that isolate PC397 reduced colonisation of or survival in the wood by DAR75532. These results were further supported by the absence of staining in the pathogen alone controls, presumably caused by the response of wood tissue to DAR75532. However, in the second experiment, conducted by Professor Giora Kritzman, DAR75532 colonies were recovered from the middle section of one twig infiltrated with PC397. It would be of interest to monitor colonisation of or survival by

DAR75532 in wood in the presence of PC397, over a period of time, to see if the pathogen could be eradicated from excised twigs and, more importantly, from living trees.

In the current experiments, the antagonist was applied before or simultaneously with DAR75532. The latter approach did not imitate the field situation where pathogen may be established first in the tree or may be introduced at pruning. A preliminary experiment was conducted by Professor Giora Kritzman to determine the effect of time of application of the antagonist on the efficacy to control DAR75532. The antagonist was infiltrated into excised pistachio twigs 5 days before or 5 days after infiltrating the same twig with suspension of DAR75532. After the last inoculation, the twigs were incubated for 10 days and the populations of DAR75532 recovered compared. When DAR75532 was applied 5 days before the antagonist, and assuming that the average populations of DAR75532 recovered from twigs infiltrated with pathogen only was 100%, the recovery was 94% from twigs infiltrated later with 64161L and 19% with PC397. The recovery of DAR75532 was much less when applied 5 days after antagonists with 0.06% when 64161L was used and 0.03% when PC397 was used. Several studies of Eutypa dieback on grapevine also reported the success of antagonists to suppress the growth of pathogen when biological control agents were applied before inoculating the wood with pathogen. Schmidt *et al*., (2001b) applied *B. subtilis* and *E. herbicola* 2 days before inoculating autoclaved grape wood with *E. lata*, and Ferreira *et al*. (1991) applied *E. lata* 4 hours after applying *B. subtilis* to pruning wounds on grapevines. However, these authors did not assess the effect of application of the antagonists after the pathogen had established in the plant. Nevertheless, the preliminary experiment in the current study, supported by the findings in the grapevine experiments, suggested that applying the antagonist before the pathogen may provide some degree of protection to the tree.

In summary, antagonists 64161L and PC397 reduced the population of DAR75532 recovered from excised pistachio twigs. Infiltrating the pathogen directly into the wood provided a more reliable method of inoculation than placing suspensions onto the wound surface. However, the latter method was sufficient to introduce the antagonists to the wood. This would be an advantage if the antagonists were to be developed further as biological control agents, as it is practical to integrate application with the pruning programme. Due to time constraints, the ability of the other six bacterial isolates used in the preliminary screening to antagonise DAR75532 in excised

twigs was not evaluated, nor the ability of the bacterial antagonists to reduce or prevent colonisation of living pistachio trees by *Xtp*.

CHAPTER FIVE

Effects of peptide BP100 on growth of *X. translucens* in liquid medium

5.1 Introduction

The potential of antimicrobial peptides against microorganisms has been widely recognised and studied (see section 1.5.5). In plant protection, a number of antimicrobial peptides has been successfully evaluated against bacterial pathogens, mainly *in vitro* (Ferre *et al*., 2006; Monroc *et al*., 2006; Oard & Enright, 2006; Badosa *et al*., 2007; Muñoz *et al*., 2007; Marcos *et al*., 2008; Badosa *et al*., 2009). There are various methods to measure the sensitivity of a bacterial pathogen to antimicrobial agents such as antibiotics and peptides. The method most frequently used *in vitro* to evaluate antimicrobial peptides is the plate-counting method, in which different concentrations of peptides are added to pathogen cell suspensions in a suitable liquid medium (Giacometti *et al*., 2000). The effect of peptide on the pathogen is then monitored by serial dilution, plating the mixtures and counting the resulting colonies. Another method is by using an automated turbidimetric system in which the optical density allows the change in biomass present or the population of microorganisms in the suspension to be measured over time (Walberg *et al*., 1996; Sánchez-Gómez *et al*., 2008). In this system, theoretically, when light penetrates through a suspension of microorganisms, the light is scattered and the amount of scatter is an indication of the biomass present in the suspension. Increase in biomass, either as cell size or density, increases turbidity. An advantage of the turbidimetric system is that it can be rapid and non-destructive (Pijls *et al*., 1994). A modification of the turbidimetric method was used in the experiments reported in this chapter.

As reported in chapter three, the composition of the growth medium affected the antimicrobial activity of the bacterial antagonists. This factor may also affect the activity of peptides, and thus an appropriate liquid medium for the determination of the peptide activity needed to be determined first. Two liquid media were tested; trypticase soy broth (TSB), which was used by Badosa *et al*. (2007) to assess the antimicrobial activity of peptides, and SPB because DAR75532 grows well in this medium.

In the *in vitro* screening, DAR75532 sometimes failed to grow on solid medium. Although all experiments reported in this chapter required the use of liquid medium, it

was considered important to examine the growth pattern of several concentrations of DAR75532 in liquid medium, and to select one concentration as the standard for the primary experiment. Two other concentrations of DAR75532 were also tested, to evaluate the effect of peptide on high and low concentrations of the pathogen.

The objective of the experiments reported in this chapter was to evaluate the effect of different concentrations of the synthetic peptide BP100 (Badosa *et al*., 2007) on the growth of DAR75532.

5.2 Materials and methods

5.2.1 Peptide

Synthetic peptide BP100 was obtained from the University of Girona, Spain (Badosa *et al*., 2007). The amino acid sequence of this peptide is H-KKLFKKILKYL-NH_2 with molecular weight of 1420.87. Stock solutions at 1000 μM were prepared by solubilising the lyophilised peptide with SDW and filtering the suspension through a sterile 0.22 μm Millipore MF membrane filter (Millex-HA). The stock solutions were kept at 5^{o}C until use.

5.2.2 Microtitre plate preparation, incubation and reading

Sterile, polypropylene flat-bottomed microtitre plates with 8 rows of 12 wells (655161, Greiner bio-one, Germany) were used in all the experiments. The suspensions were dispensed with an 8-channel pipettor (Finnpipette®, Thermo Scientific, Finland) using aseptic technique. To obtain an even growth of pathogen cells in the wells, DAR75532 suspension was dispensed first before adding the liquid medium and the peptide. The plates were then sealed with Polyprop-adhesive film to prevent contamination and spillage. The microtitre plates were incubated at 28^{o}C with continuous shaking at 80 rpm throughout the experiment.

Optical density (OD) of the bacterial culture was measured at 600 nm with a spectrophotometer (SpectraMAX 250, Molecular Devices Ltd, USA). With this device, the plastic seal of the microtitre plates needed to be removed during the reading to allow accurate measurement of bacterial population in the suspension. This resulted in the loss of sterile environment in the corresponding wells, which would affect the accuracy of

the result. Therefore, each treatment was replicated by the number of readings that had to be taken over time. At each reading time, the plastic seal was removed from the top of the wells from which the OD was read, leaving the neighbouring wells sealed and protected from contaminants. Immediately before measurement, the plates were agitated on a shaker set at 300 revolutions/minute for about 60 seconds to avoid sedimentation in the wells.

5.2.3 Growth performance of DAR75532 from two inoculum concentrations in two culture media

A preliminary experiment was conducted to determine a suitable medium for assessment of the peptide. The growth performance of DAR75532 in TSB and SPB from two inoculum concentrations (10^4 and 10^6 CFU/ml) was monitored. DAR75532 at 10^6 CFU/ml was selected for the high concentration because a similar concentration was used in earlier experiments and 10^4 CFU/ml to represent a low concentration of the pathogen.

DAR75532 suspensions were prepared by serial dilution as described in section 2.2. Forty µl aliquots from each concentration were mixed with 160 µl of either TSB or SPB, and each medium mixed with 40 µl SDW was used for controls. Each concentration of DAR75532 in each medium was replicated four times with odd-number columns in two different rows as replicates 1 and 3, even-number columns in two different rows as replicates 2 and 4 (Figure 5.1). Treatments were replicated in three other microtitre plates and OD was recorded at 12, 14, 16, 18, 20, 36, 38, 40, 42, 44, 60, 62, 64, 66 and 68 hours, as described in section 5.2.2.

5.2.4 Growth performance of DAR75532 from different inoculum concentrations

For this and subsequent experiments, SPB was chosen as DAR75532 grew most consistently in this medium (experiment 5.2.3). This preliminary assessment was conducted to determine a standard concentration of DAR75532 for use in the primary experiments. This was done by assessing the growth performance from seven inoculum concentrations of DAR75532 in SPB.

Suspensions of DAR75532 at the concentrations of 10^1 to 10^7 CFU/ml were prepared in SDW (as described in section 2.2). Forty µl of each concentration were

dispensed into each well, before adding 160 µl SPB. DAR75532 suspension at an initial concentration of 10^7 CFU/ml was assigned to row A, 10^6 CFU/ml to row B and so on (Figure 5.2). Controls consisted of 40 µl SDW in 160 µl SPB. For each reading time, each concentration was replicated four times, allowing three reading times per plate. Suspensions were distributed in two microtitre plates, so that there were sufficient replicates for six measurements. Growth of DAR75532 was assessed by measuring OD at 0, 8, 24, 32, 48 and 56 hours as described in section 5.2.2. In this experiment, the first OD measurement was taken at 0 hour and not at 12 hours as in the previous experiment (section 5.2.3).

5.2.5 Effect of peptide BP100 on 10^6 CFU/ml of DAR75532

The ability of synthetic peptide BP100 to suppress the growth of DAR75532 was investigated by mixing equal volume of peptide and DAR75532, incubating them in SPB at 28°C and monitoring the OD of the resulting suspensions.

A method modified from Badosa *et al*. (2007) was applied in this experiment. A stock solution of peptide BP100 at 1000 µM (section 5.2.1) was diluted to 25, 50 and 100 µM in SDW. Twenty µl of each concentration of peptide were mixed with 20 µl of DAR75532 suspension at 10^6 CFU/ml and 160 µl SPB, giving final concentrations of peptide of 2.5, 5 and 10 µM. All suspensions were dispensed directly into microtitre plates as shown in Figure 5.3. All treatments using the different concentrations of peptides were replicated four times. The positive controls were 20 µl DAR75532 suspension mixed with 20 µl SDW in 160 µl SPB and the negative controls were peptide at the above concentrations in SPB. The negative controls were replicated twice and the whole experiment was repeated. For each experiment, four plates were prepared to allow OD readings to be taken at 0, 16, 18, 20, 22, 24, 40, 42, 44, 46 and 48 hours, as described in section 5.2.2.

To assess the viability of DAR75532 at 48 hours, 100 µl of each suspension were taken from each sealed column (columns 9 to 12 of the fourth microtitre plates) and spread evenly on SPA. The plates were then incubated at 28°C in the dark and monitored daily from 48 hours to 14 days of incubation. Plates with growth of DAR75532 growth were considered positive.

5.2.6 Effect of peptide BP100 on 10^8 CFU/ml of DAR75532

In the above experiment, the peptide at the lowest concentration tested seemed to suppress growth of DAR75532 at 10^6 CFU/ml in SPB, but the bacterium produced colonies when transferred to solid medium. The effect of the same concentration of peptide on a higher concentration of the pathogen was then examined. An experiment similar to the one above was set up, using DAR75532 at 10^8 CFU/ml and peptide at a final concentration of 2.5 μM. The treatment was replicated four times and the controls were replicated twice. OD was recorded as above and aliquots of 100 μl were taken from each sealed column (9, 10, 11 and 12) of the fourth microtitre plate, and spread evenly on SPA. The plates were then incubated at 28^{o}C in the dark and growth of DAR75532 was assessed daily from 48 hours up to 14 days.

5.2.7 Effect of peptide BP100 on 10^4 CFU/ml of DAR75532

To evaluate the effect of peptide BP100 on a lower concentration of DAR75532, which might reflect a more natural inoculum dose (Sedgley *et al.*, 2006; Taylor *et al.*, 2007), a similar experiment was carried out. Due to limited availability of peptide, only two concentrations of peptide at 2.5 and 5 μM were assessed against DAR75532 at 10^4 CFU/ml. Each treatment was replicated four times and OD was recorded at 0, 16, 24, 40 and 48 hours. There were four replicates of the positive (DAR75532 alone) and two of the negative (peptide alone) controls. A volume of 100 μl was removed at 48 hours from columns 9 to 12 of the second microtitre plate and spread evenly on SPA to monitor growth of DAR75532, as described above.

5.2.8 Assessment of peptide BP100 on DAR75532 by plate counting method

Peptide BP100 was observed to be bactericidal to DAR75532 (10^6 CFU/ml) at 5 and 10 μM, and bacteriostatic at 2.5 μM (see section 5.3.3). An experiment based on plate counts was carried out to verify the bactericidal activity of peptide BP100 observed in the previous experiments. An assay adapted from Badosa *et al*. (2007) was used. The effectiveness of the peptide to kill DAR75532 was determined by comparing the colony numbers recorded with the untreated control. DAR75532 suspension was serially diluted to 10^6 CFU/ml in SPB (as described in section 2.2). Peptide BP100 was added to the bacterial suspension to give final concentrations of 5 and 10 μM in a total volume of 3 ml in a 10 ml sterile centrifuge tube. Controls were bacterial suspension

without peptide. The mixed suspensions of DAR75532 and peptide were incubated at 28^{o}C with continuous shaking for 3 hours. To determine the viability or survival of DAR75532 cells, aliquots of 300 µl were removed from the tubes at 30-minute intervals, starting at the beginning of the incubation time (0 hour), and diluted 20-fold. Aliquots of 100 µl were transferred to SPA and incubated at 28^{o}C. After 48 hours, the colony forming units were enumerated. The plates were examined for up to 14 days to monitor the bactericidal effect of the peptide. Treatments were replicated four times and the whole experiment was repeated.

5.2.9 Statistical analysis

Each set of experiments (5.2.3 to 5.2.8) in this study was conducted twice and analysed individually but only results from the first experiments are presented as the repetition produced a statistically identical outcome. The mean values for OD were then tested for significant differences by analysis of variance, and the least significant difference test was used to compare treatment means at $P<0.05$.

5.3 Results

5.3.1 Growth performance of DAR75532 from two inoculum concentrations in two culture media

The growth performance of DAR75532 from inoculum concentrations of 10^4 and 10^6 CFU/ml in SPB and TSB is shown in Figure 5.4. The OD for SDW in both SPB and TSB (controls) was almost constant except in SPB after 62 hours of incubation. The OD of the SPB controls was higher than that of the TSB controls. Two-way analysis of variance indicated that there was significant difference between OD for each time point among the treatments (LSD=0.005 at $P<0.05$). However, there was no difference in the growth pattern of DAR75532 from both concentrations. Generally, DAR75532 at both inoculum concentrations in SPB generated higher OD readings than in TSB. At the first recording after 12 hours of incubation, the OD was slightly higher in DAR75532 at inoculum concentration of 10^6 CFU/ml than in 10^4 CFU/ml. The pattern of growth curves produced by DAR75532 cells at 10^4 CFU/ml in both media was similar, but for 10^6 CFU/ml of DAR75532, the OD declined after 48 hours of incubation in TSB. Some variation occurred among replicate wells, especially in TSB at the longer incubation periods (Figure 5.4).

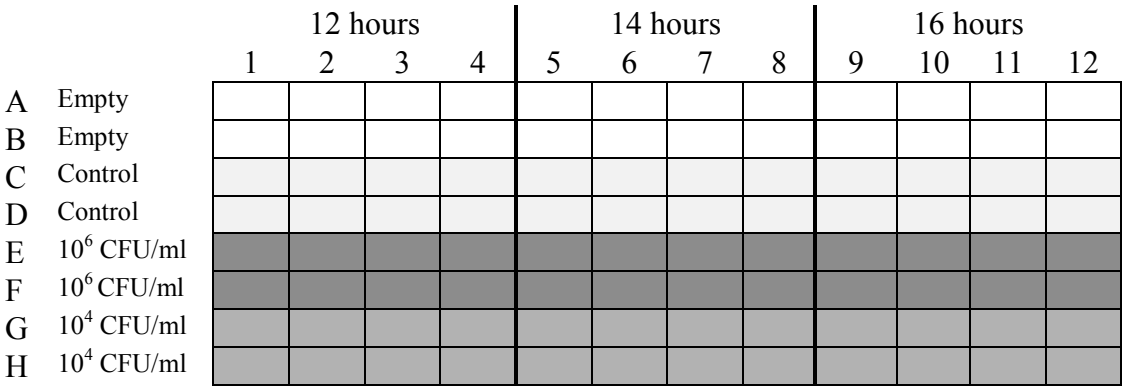


Figure 5.1: Layout of Experiment 5.2.3; growth of DAR75532 from two inoculum concentrations in SPB and TSB. TSB was dispensed in columns 1, 2, 5, 6, 9 and 10; the rest of the columns received SPB. DAR75532 at initial concentration of 10^6 CFU/ml was assigned to rows E and F; 10^4 CFU/ml was assigned to rows G and H; controls to rows C and D. OD for columns 1 to 4 was recorded at 12 hours, columns 5 to 8 at 14 hours and 9 to 12 at 16 hours. At 18, 20 and 36 hours, readings were taken from a second microtitre plate, with pattern of data recording similar to the first microtitre plate. OD measurements at 38, 40 and 42 hours were taken from a third microtitre plate; and 44, 60 and 62 hours from a fourth plate.

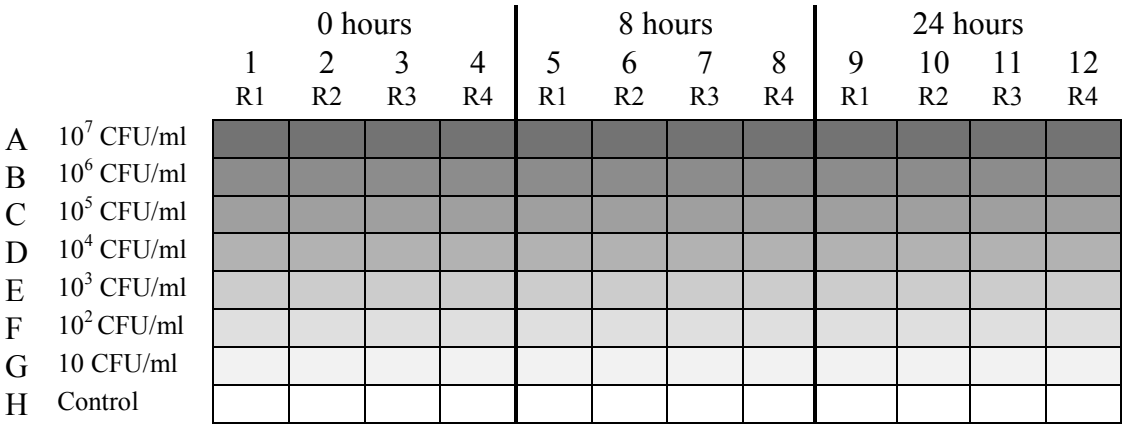


Figure 5.2: Layout of Experiment 5.2.4; growth of DAR75532 in SPB from a series of inoculum concentrations. Rows A to G each contained DAR75532 at different initial concentrations (from 10 to 10^7 CFU/ml) and the negative controls were assigned in row H. OD for columns 1 to 4 was recorded at 0 hour; columns 5 to 8 at 8 hours; 9 to 12 at 24 hours. At 32, 48 and 56 hours, readings were taken from a second microtitre plate, with pattern of data recording similar to the first microtitre plate. R=replicate.

		0 hours				16 hours				18 hours			
		1	2	3	4	5	6	7	8	9	10	11	12
A	100 μM	✧	✧			✧	✧			✧	✧		
B	100 μM	★	★			★	★			★	★		
C	50 μM	✧	✧			✧	✧			✧	✧		
D	50 μM	★	★			★	★			★	★		
E	2.5 μM	✧	✧			✧	✧			✧	✧		
F	2.5 μM	★	★			★	★			★	★		
G	SPB												
H	SPB												

Figure 5.3: Layout of Experiment 5.2.5; growth of DAR75532 (at initial inoculum of 10^6 CFU/ml) in SPB treated with three concentrations of peptide BP100. ✧ represents DAR75532 in SPB (positive controls), ★ represents peptide in SPB (negative controls). Rows A and B, excluding wells marked ✧, were filled with peptide at 100 μM, rows C and D with peptide at 50 μM, rows E and F with 25 μM. Rows G and H contained SPB only. OD for columns 1 to 4 was recorded at 0 hour; columns 5 to 8 at 16 hours; columns 9 to 12 at 18 hours. At 20, 22 and 24 hours, readings were taken from a second microtitre plate, with pattern of data recording similar to the first microtitre plate. OD measurements at 40, 42 and 44 hours were taken from a third microtitre plate; and 46 and 48 hours from a fourth plate.

5.3.2 Growth performance of DAR75532 from different inoculum concentrations

The growth performance of DAR75532 from seven inoculum concentrations is shown in Figure 5.5. In the un-inoculated SPB, the OD was constant. Generally, DAR75532 inoculated into SPB at concentrations above 10^2 CFU/ml generated a typical bacterial growth curve (Figure 5.5). At 10^2 CFU/ml and below, there was little evidence of growth. The duration of the lag phase differed for each inoculum concentration, and increased as the initial concentration of cells decreased. The duration of the exponential phase was about 32 hours for an initial concentration of 10^7 CFU/ml, and 48 hours when the initial concentration was 10^6 CFU/ml. When inoculated at 10^3 to 10^5 CFU/ml, DAR75532 cells did not reach the end of the exponential phase before 56 hours. The highest OD value reached was 0.206 after 32 hours of incubation from an initial concentration of 10^7 CFU/ml. From 10^6 CFU/ml, DAR75532 reached a maximum OD of 0.205 after 48 hours of incubation.

Even though there is a significant difference between the optical density readings for each time point among inoculum concentrations ($P<0.05$), there was no difference in the pattern of growth of DAR75532 at concentrations of 10^3 CFU/ml and above (Figure 5.5). Therefore, to maintain consistency with the previous preliminary screening, DAR75532 at 10^6 CFU/ml was chosen as the standard concentration for the primary experiments.

5.3.3 Effect of peptide BP100 on 10^6 CFU/ml of DAR75532

The effect of three concentrations of peptide BP100 on the growth of DAR75532 from an initial concentration of 10^6 CFU/ml is presented in Figure 5.6. The growth curve for DAR75532 from this concentration in peptide-free SPB (positive control) was similar to that obtained in previous experiments. The highest OD reading (0.285) was reached at about 48 hours of incubation. The OD for the un-inoculated peptide suspension (negative controls) was almost constant except in 2.5 μM peptide, where there was a slight increase in the OD at 18 hours. Peptide BP100 at the three concentrations evaluated seemed to suppress the growth of DAR75532 in SPB as OD readings over 48 hours were similar to those in the negative controls. The growth of DAR75532 was significantly reduced (LSD=0.0034 at $P<0.05$) by all the three concentrations of peptide tested compared with the positive control.

When aliquots of bacterial suspension sampled after 48 hours of incubation in the presence of BP100 at 2.5 μM were spread on SPA, however, plates were covered with lawn of the pathogen, similar to the untreated control (Figure 5.7). For suspensions incubated with 5 μM of BP100, only three colonies of DAR75532 were recovered on one replicate plate and none on the other three replicates. At the highest concentration evaluated (10 μM), 53 colonies of DAR75532 were recovered on one replicate plate only.

5.3.4 Effect of peptide BP100 on 10^8 CFU/ml of DAR75532

The effect of peptide BP100 at 2.5 μM on growth of DAR75532 from an initial concentration of 10^8 CFU/ml in SPB is shown in Figure 5.8. Untreated DAR75532 produced a growth curve similar to a typical bacterial growth curve. The OD reading obtained from un-inoculated peptide in SPB was constant. Although there was a significant difference between the OD for each time point between the treatment and positive control (LSD=0.0041 at $P<0.05$), there was no overall difference in the pattern of growth of DAR75532 treated with 2.5 μM peptide and in un-treated DAR75532. Initially, the peptide appeared to delay the growth of DAR75532 but, after 22 hours of incubation, the combination of DAR75532 and 2.5 μM peptide exhibited higher OD readings ($P<0.05$) than the untreated control (DAR75532 in SPB). When aliquots were spread on SPA after 48 hours of incubation, a lawn of pathogen was observed on all replicate plates, but was not as dense as in the positive controls (Figure 5.9).

5.3.5 Effect of peptide BP100 on 10^4 CFU/ml of DAR75532

The effect of peptide BP100 at 2.5 μM on growth of DAR75532 from an initial concentration of 10^4 CFU/ml in SPB is shown in Figure 5.10. The pattern of growth curve generated by untreated DAR75532 from an initial concentration of 10^4 CFU/ml was similar to that obtained in experiment 5.3.2. Un-inoculated peptide at both 2.5 and 5 μM produced constant OD readings. Peptide at both concentrations evaluated suppressed growth of DAR75532 in the liquid medium compared with the positive control (LSD=0.0033 at $P<0.05$). After incubation for 40 hours, the combination of DAR75532 and 2.5 μM peptide gave a slight increase in OD value, but declined again at 48 hours. When sampled at 48 hours of incubation, there was no growth of DAR75532 on SPA following exposure to 5 μM peptide, whereas at 2.5 μM peptide,

DAR75532 colonies were recovered from two of the four replicates, but the number of colonies were very much less than in the positive control (Figure 5.11).

5.3.6 Assessment of peptide BP100 on DAR75532 by plate counting method

The bactericidal activity of peptide BP100 at 5 and 10 μM on the growth of DAR75532 from 10^6 CFU/ml is illustrated in Figure 5.12. Peptide at different concentrations had different effects on the growth of DAR75532. Over time, 10 μM peptide killed DAR75532 cells faster than at 5 μM. At 3 hours of incubation, 10 μM killed 97% of DAR75532, and 5 μM killed about 77% of cells. Peptide at both concentrations started to kill DAR75532 cells the moment it was applied to DAR75532 suspensions. Immediately after inoculation, peptide at 5 μM killed about 53% of DAR75532 cells and the percentage of cell mortality was about 62% when 10 μM was applied (Figure 5.12). This percentage was obtained by comparing the colony numbers produced by the treated suspensions and the untreated control.

5.4 Discussion

In the absence of peptide BP100, cultures of DAR75532 inoculated into SPB or TSB generally resulted in a typical bacterial growth curve. Generally, there are four stages distinguished in the typical bacterial growth curve (Swinnen *et al*., 2004), beginning with the lag phase immediately after inoculation of cells into fresh medium. Second is the exponential stage where the bacterial cells undergo binary fission and the population doubles in number every generation time. In the third stage, the stationary stage, the bacteria do not multiply any more and at the final stage the cells die or undergo lysis. In the current study, growth of DAR75532 was compared with an un-inoculated SPB control. The OD readings for the controls were more or less constant throughout the period of incubation, indicating that contamination was unlikely to have occurred. The small increase in the OD value for the SPB control at 62 hours in experiment 5.3.1 is considered more likely to reflect operator error than microbial contamination.

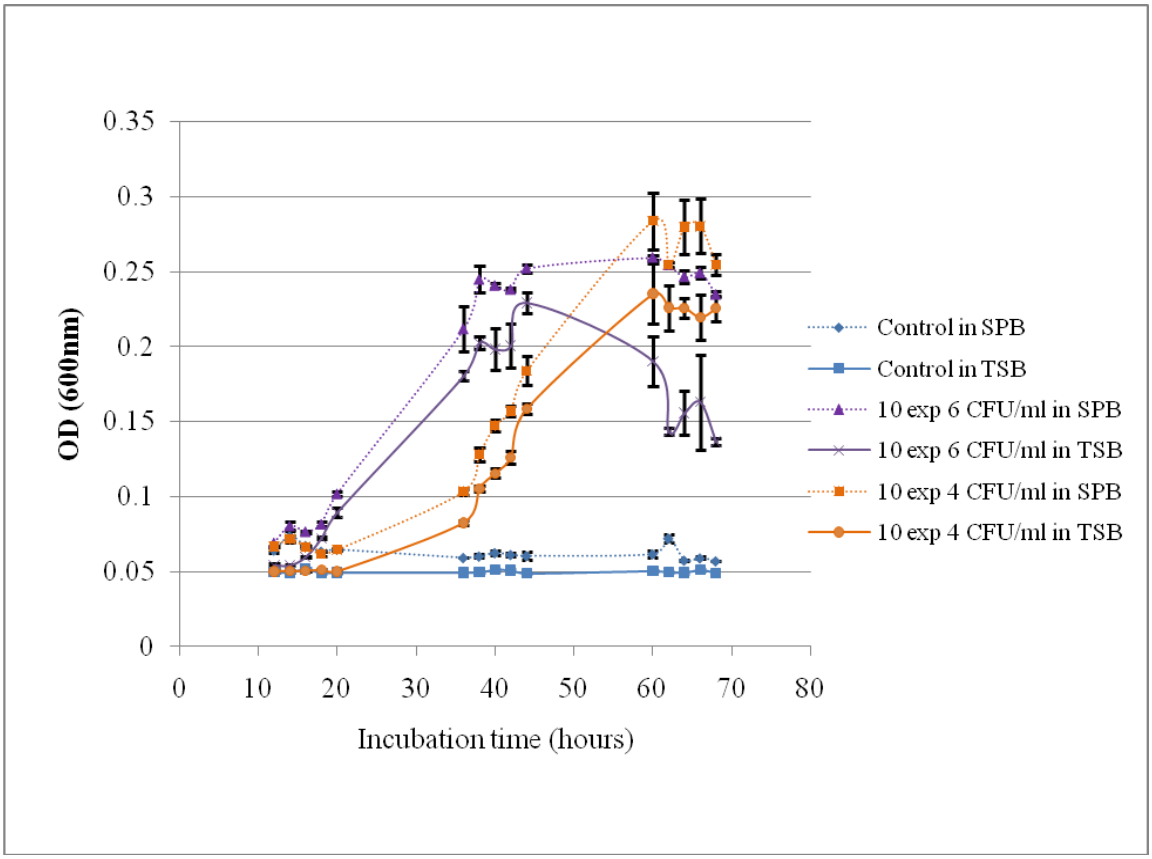


Figure 5.4: Growth curve of DAR75532 from inoculum concentrations of 10^4 and 10^6 CFU/ml in SPB and TSB, assessed by OD measurements at 600 nm over time. Controls comprised SDW in either SPB or TSB. Each point is the mean of four replicates ± SE.

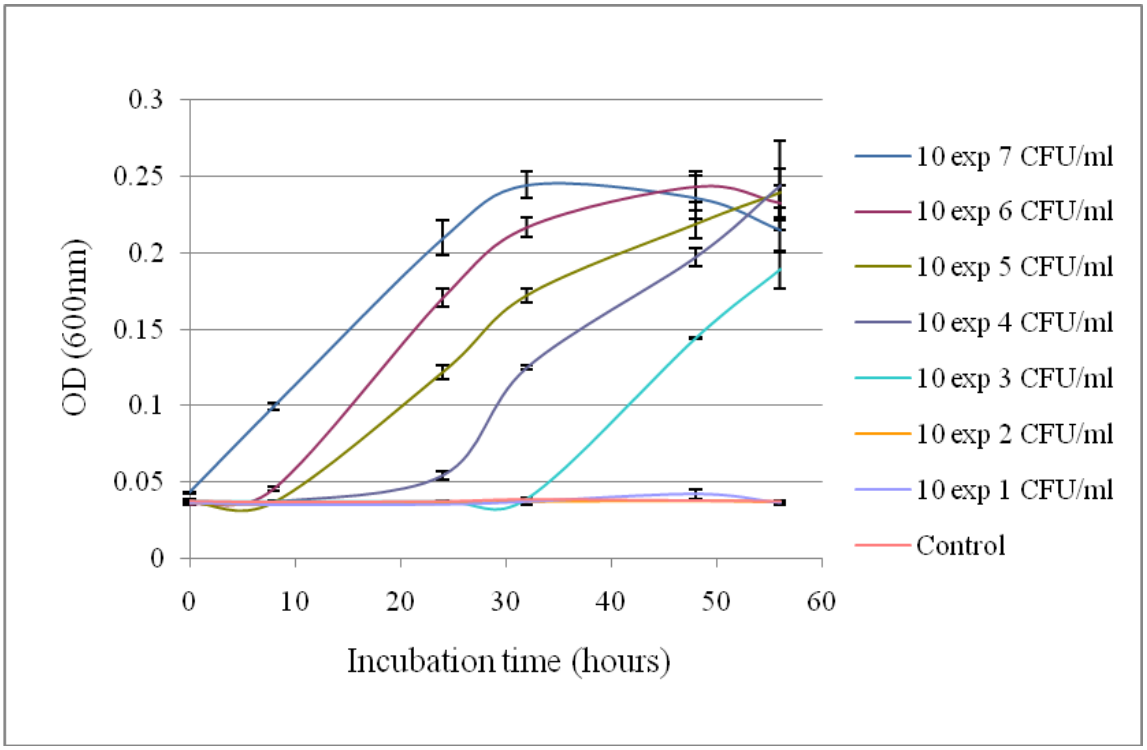


Figure 5.5: Growth curve of DAR75532 from different inoculum concentrations (from 10 to 10^7 CFU/ml) in SPB, in terms of OD at 600 nm. The control comprised SDW in SPB. Each point is the mean of four replicates ± SE.

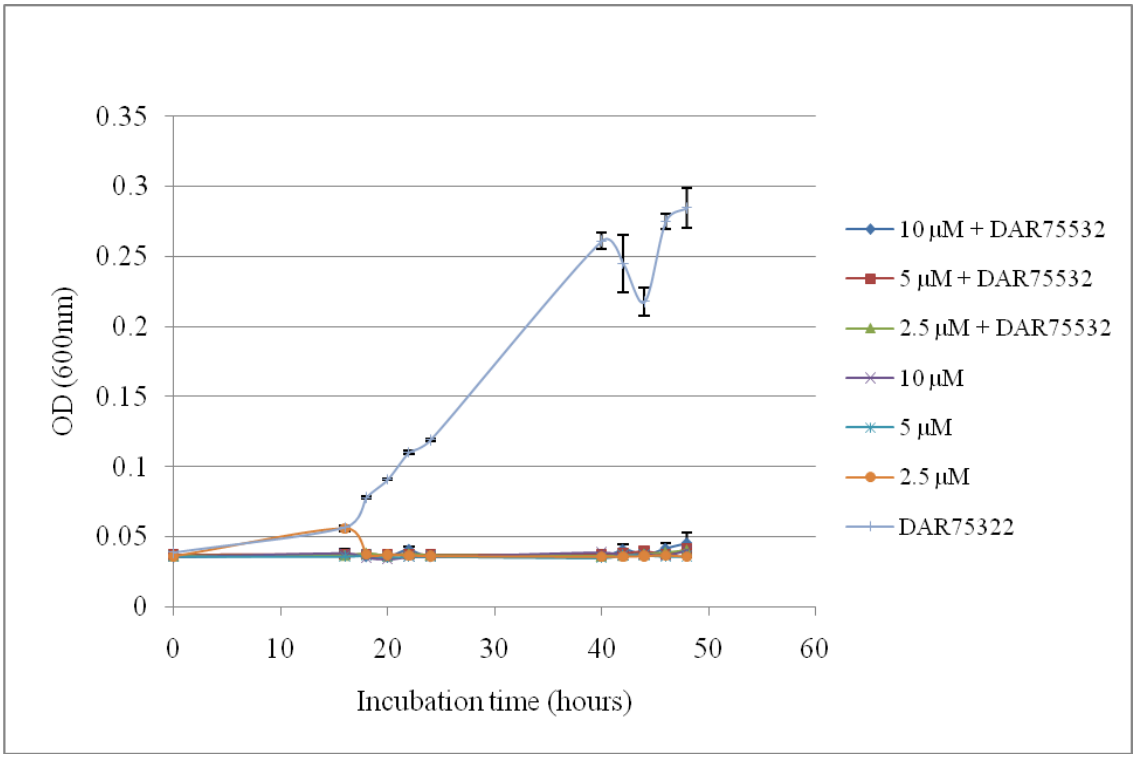
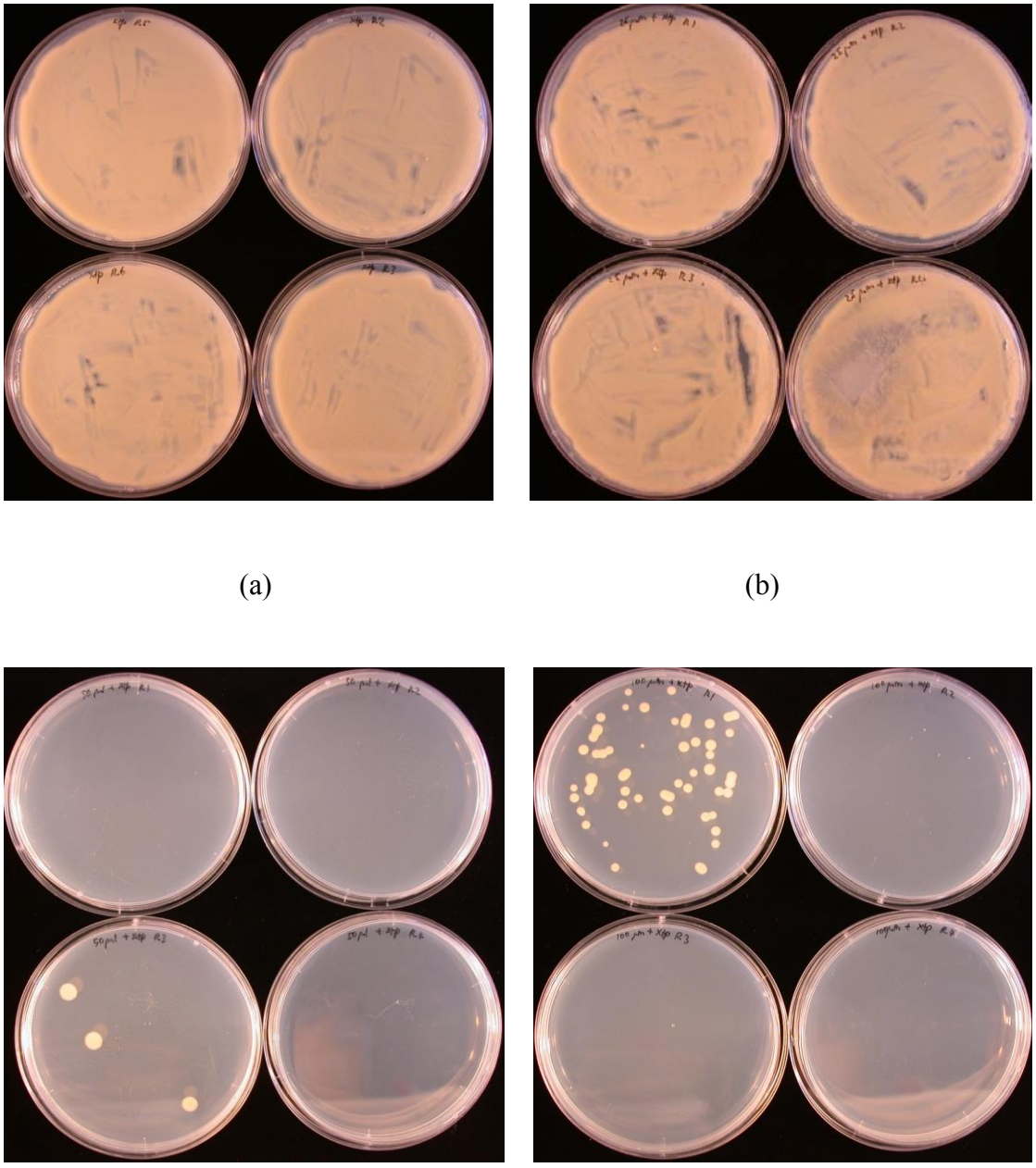


Figure 5.6: Effect of peptide BP100 at three concentrations (2.5, 5 and 10 µM) on growth of DAR75532 in SPB from an initial concentration of 10^6 CFU/ml, assessed by OD at 600 nm. The positive control comprised DAR75532 in SPB, and negative controls were un-inoculated peptide BP100 at the three different concentrations in SPB. Each point is the mean of four replicates ± SE, in the treatments and positive controls.



(a) (b)

(c) (d)

Figure 5.7: Effect of peptide BP100 at three concentrations on DAR75532 (from an initial concentration of 10^6 CFU/ml) assessed by plating after 48 hours of incubation in SPB. Aliquots of 100 µl were sampled and spread on SPA. (a) Untreated DAR75532 (positive control), plates from all replicates were covered with a lawn of DAR75532; (b) DAR75532 treated with 2.5 µM peptide, plates from all replicates were covered with a lawn of DAR75532; (c) DAR75532 treated with 5 µM peptide, three colonies of DAR75532 were recovered on one replicate plate; (d) DAR75532 treated with 10 µM peptide, 53 colonies of DAR75532 were recovered on one replicate plate.

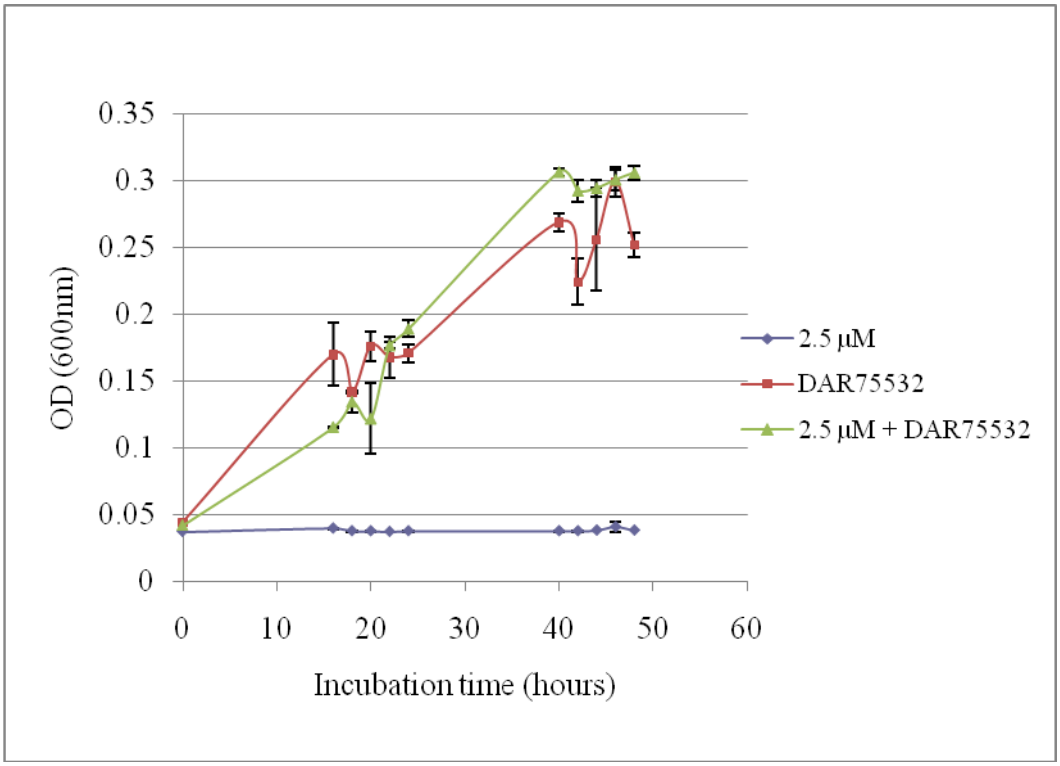
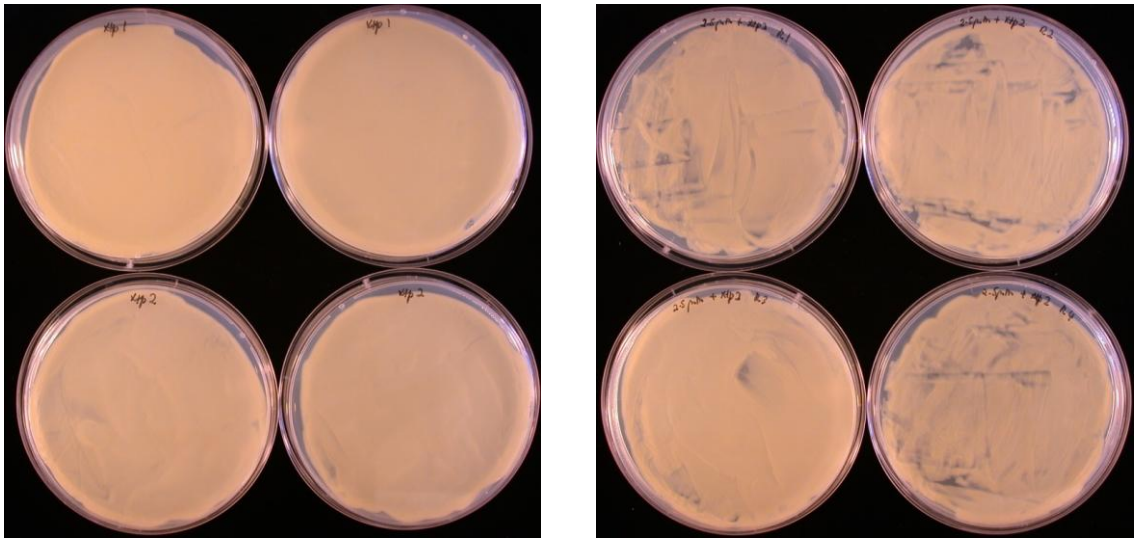


Figure 5.8: Effect of peptide BP100 at 2.5 µM on the growth of DAR75532 in SPB from an initial concentration of 10^8 CFU/ml, assessed by OD at 600 nm. The positive control comprised untreated DAR75532 at 10^8 CFU/ml in SPB, and the negative control was un-inoculated peptide BP100 at 2.5 µM in SPB. Each point is the mean of four replicates ± SE in the treatment and positive controls.



(a) (b)

Figure 5.9: Effect of peptide BP100 at 2.5 μM on DAR75532 (from an initial concentration of 10^8 CFU/ml), assessed by plating, after 48 hours of incubation in SPB. Aliquots of 100 μl were sampled and spread on SPA. (a) Untreated DAR75532 (positive control), plates from all replicates were covered with a lawn of DAR75532; (b) DAR75532 treated with 2.5 μM peptide, a lawn of DAR75532 grew on all replicate plates.

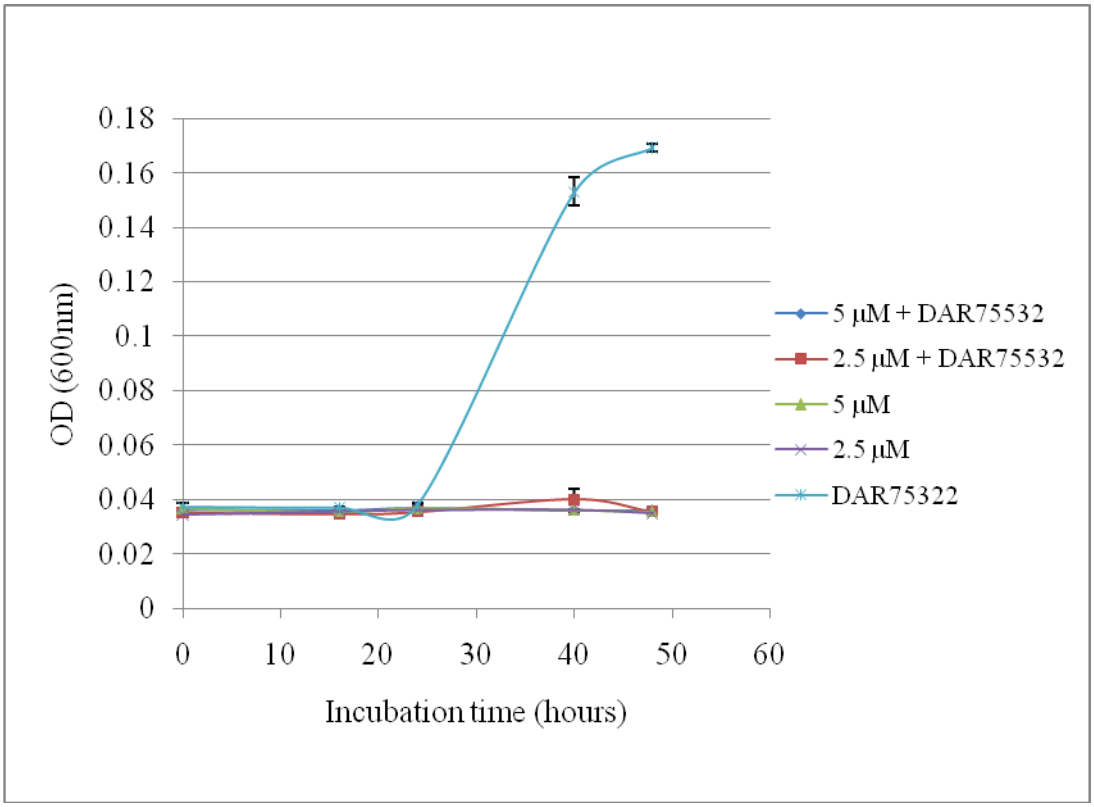
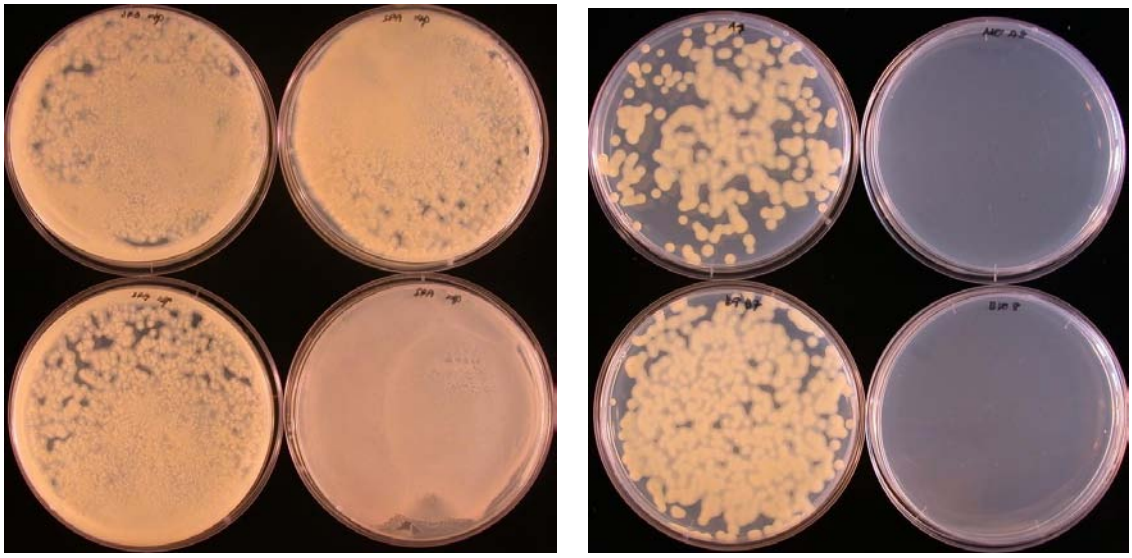
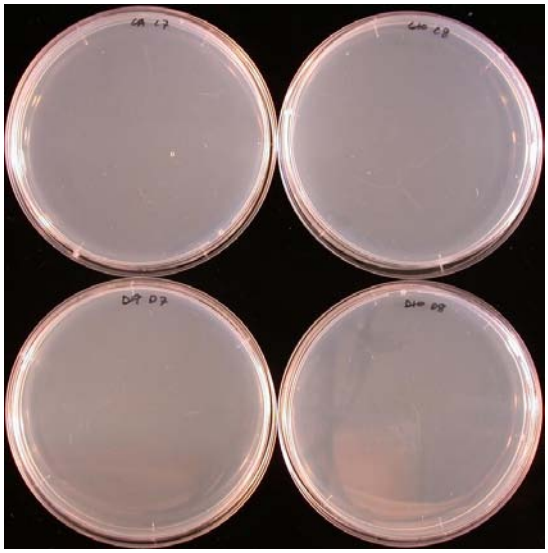


Figure 5.10: Effect of peptide at 2.5 and 5 µM on the growth of DAR75532 from an initial concentration of 10^4 CFU/ml, assessed by OD at 600 nm. The positive control comprised untreated DAR75532 at 10^4 CFU/ml in SPB, and the negative control was un-inoculated peptide BP100 at 2.5 and 5 µM in SPB. Each point is the mean of four replicates ± SE in the treatments and positive controls, and two replicates ± SE in the negative controls.



(a) (b)



(c)

Figure 5.11: Effect of peptide BP100 at 2.5 and 5 µM on DAR75532 (from an initial concentration of 10^4 CFU/ml), assessed by plating, after 48 hours of incubation in SPB. Aliquots of 100 µl were sampled and spread on SPA (a) Untreated DAR75532 (positive control), plates from three replicates were covered with a lawn of DAR75532 and contamination occurred in the fourth replicate (bottom right), (b) DAR75532 treated with 2.5 µM peptide, DAR75532 colonies were recovered on two replicate plates, (c) DAR75532 treated with 5 µM peptide, DAR75532 was not recovered.

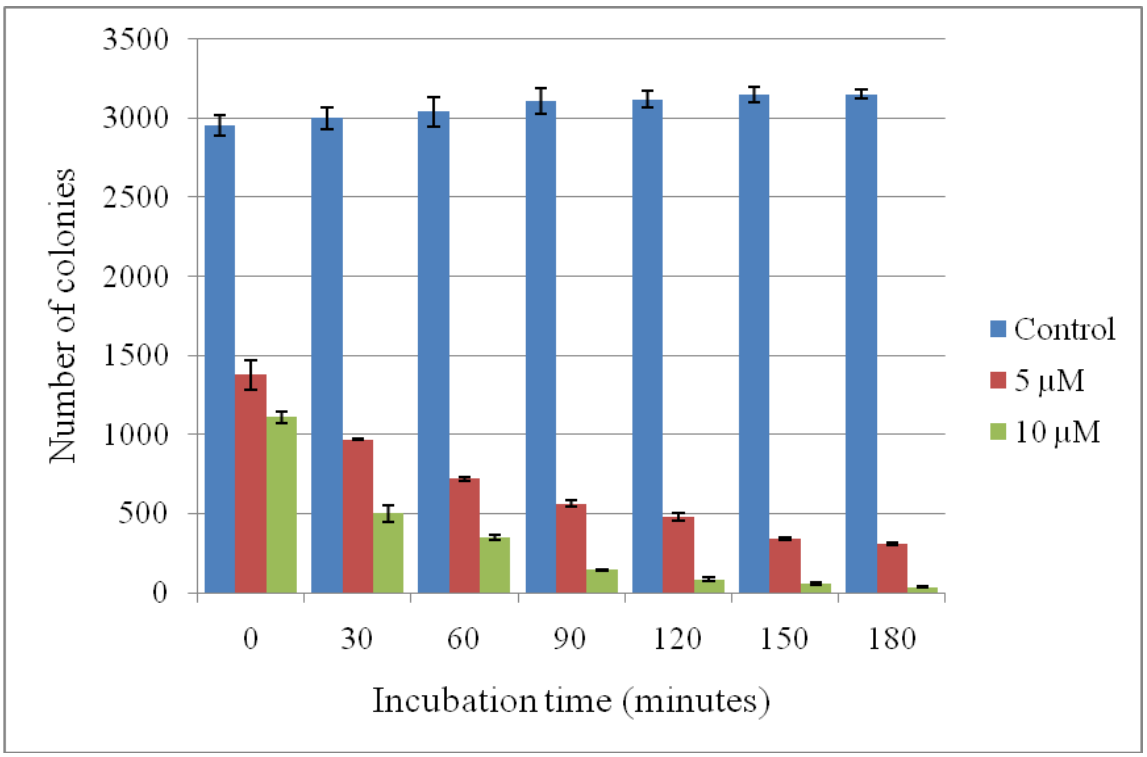


Figure 5.12: Number of DAR75532 colonies produced on SPA when mixed with 5 and 10 µM peptide BP100, and incubated for 180 minutes. Each point is the mean of four replicates ± SE.

DAR75532 grown in the two culture media resulted in different OD values over time. The OD for DAR75532 grown in SPB was generally higher than for cultures in TSB. This was the case for the un-inoculated media also, SPB being more viscous and therefore more optically dense than TSB. DAR75532 from both initial concentrations seemed to grow better in SPB than in TSB, especially as the culture aged; the OD readings for DAR75532 grown in TSB inoculated with 10^6 CFU/ml declined rapidly after a short stationary phase. These observations suggested that the nutrient composition of the liquid medium affected the growth of DAR75532, particularly in the later stages of growth, and led to the selection of SPB for subsequent experiments.

The duration of the lag phase differed for each inoculum concentration of DAR75532 tested in SPB. Initial concentration and other factors such as incubation temperature and condition of the cells (that is, whether the cells are injured or not) are known to influence the growth of other bacteria (Swinnen *et al*., 2004). At high initial cell density, DAR75532 adapted and multiplied rapidly whereas at lower initial concentrations, the pathogen cells seemed to take longer to initiate multiplication. The composition of the medium is believed to contribute to this phenomenon (Haapalainen *et al.*, 2000), although the composition of SPB and TSB did not seem to affect growth of DAR75532 as observed in experiment 5.3.1, where the duration of the exponential phase was similar in both media. DAR75532 cells reached death phase earlier when the initial concentration was high. This observation can only be applied to DAR75532 at 10^7 and 10^6 CFU/ml as readings for cultures initiated from other concentrations ceased before the cultures reached the stationary phase. OD measurements at longer than 56 hours for all initial concentrations tested would be useful for comparison of the growth curves of DAR75532. The culture initiated from 10 and 10^2 CFU/ml did not seem to grow during the incubation period. It is possible that DAR75532 cells were not identically distributed in each well, and at this concentration, very few cells (four for 10^2 and 0.4 for 10^1) would be distributed into each well, and some wells may not have received cells at all. In those wells that receive bacteria, the multiplication of individual cell depends on other bacterial cells by communicating with each other and release hormones or pheromones for growth (Kaprelyants & Kell, 1996), and the cells may require longer than 56 hours to grow to a detectable concentration. However, different isolates of *Xtp* may have different growth patterns and variation within the pathovar should be evaluated.

Overall, the addition of BP100 to cultures of DAR75532 in SPB inhibited the growth of the pathogen. This finding is in agreement with the report by Badosa *et al*. (2007) that BP100 inhibited the growth of the plant pathogenic bacteria, *X. axonopodis*, *E. amylovora* and *P. syringae*. The antibacterial effect of this peptide on DAR75532 was influenced by the initial inoculum concentration of the pathogen. In the present study, BP100 at 2.5 µM was sufficient to suppress the growth of DAR75532 from initial concentrations of 10^4 and 10^6 CFU/ml, but insufficient when the initial concentration was 10^8 CFU/ml. Badosa *et al*. (2007) also reported that BP100 at a range of 5.0 to 7.5 µM exhibited antibacterial activity against *X. axonopodis*, although the initial concentration of the pathogen was not specifically mentioned in their work. So far, the influence of inoculum concentration on the efficacy of peptides has not been reported but the effect of the fungicide ketoconazole on the yeast *Candida albicans* was reported to be inoculum-dependent (Stevens, 1984), as was the effect of the antibiotic metronidazole on the human pathogen *Helicobacter pylori* (Hartzen *et al*., 1997). In addition to the inoculum concentration, the antimicrobial activity of peptides may also be affected by the medium used (Hartzen *et al*., 1997; Badosa *et al*., 2009). However, this factor was not investigated in these experiments. Other incubation factors, such as pH and temperature, could also influence the activity of BP100 and these should be considered in future research.

The effect of peptide BP100 on DAR75532, monitored through spectrophotometer readings, suggested that all peptide concentrations evaluated inhibited the growth of DAR75532 from inoculum of 10^6 CFU/ml. But when aliquots treated with 2.5 µM were transferred to SPA, growth of DAR75532 was evident on the plates. Therefore, at this concentration of DAR75532, BP100 at 2.5 µM seemed to have a bacteriostatic effect on the pathogen cells. However, the effect was bactericidal when these suspensions were treated with higher concentrations of the peptide. BP100 at 5 µM was bactericidal when the inoculum concentration of DAR75532 was 10^4 CFU/ml. In the plate-count experiment, the bactericidal effect of BP100, as loss of viability, was observed as soon as the cells were exposed to the peptide. Within 30 minutes of incubation, the mortality of DAR75532 was 68% when treated with 5 µM peptide. At the same concentration of BP100, 99% of *X. axonopodis* cells were killed within 30 minutes of incubation (Badosa *et al*., 2007). This indicated that DAR75532 was less sensitive to BP100 than *X. axonopodis,* however, the two species would need to be compared in the same experiment before any conclusions can be made. Furthermore, isolates of *Xtp* may differ in response to BP100 so a range of isolates should be

compared. Bactericidal activity is preferable to bacteriostatic activity as a control strategy as the effect of the former persists for longer after application of the antimicrobial agent (Montesinos & Bardají, 2008).

Minimum Inhibitory Concentration (MIC) is the lowest concentration of an antimicrobial compound, such as a peptide, which inhibits the growth of the test microorganism (Lehtinen *et al*., 2006; Sánchez-Gómez *et al*., 2008). It is commonly used to quantify the efficacy of peptides. Although the MIC of peptide BP100 to inhibit DAR75532 at 10^4 and 10^6 CFU/ml in the current study was in the range of 2.5 to 10 μM, the MIC needs to be determined more precisely by testing a wider range of peptide concentrations. However, the differences in efficacy observed here suggested that MIC is dependent on the inoculum concentration, so this needs to be considered.

As well as MIC, minimal bactericidal concentration (MBC) is also used to measure the response of target pathogen cells to antibacterial agents such as peptides or antibiotics. MBC is defined as the lowest concentration of a peptide, drug or antibiotic concentration that kills 99.9% of the initial inoculum (Tam *et al*., 2005; Lehtinen *et al*., 2006; Sánchez-Gómez *et al*., 2008). It is normally determined by the plate-count technique. In the experiments conducted in the present study, the bactericidal effect of BP100 on DAR75532 was clearly demonstrated by the reduction in the percentage of viable cells. The MBC against DAR75532 at 10^6 CFU/ml estimated from this experiment was 10 μM, even though at this concentration, the peptide killed only 97% of the bacteria. The experiment should be repeated with a range of peptide concentrations around 10 μM and various concentrations of DAR75532 to determine the conditions required to achieve 99.9% mortality of the bacterial population.

The precise mode of action of peptide BP100 against DAR75532 was not investigated in this study. Generally, peptides are thought to inhibit growth of Gram-negative bacteria by binding to lipopolysaccharide which is present in the outer membrane and penetrating or disrupting the cell membrane (Andrä *et al*., 2006). This mechanism was also reported by Badosa *et al*. (2007) in their study on the effect of BP100 on *X. axonopodis*, *E. amylovora* and *P. syringae*. Other peptides, such as PEP3, exhibited a similar mechanism when evaluated on the same three species (Ferre *et al*., 2006). However, most research on this topic has focused on medically important bacteria. For example Park *et al*. (1998) examined the mode of action of peptides on *Escherichia coli* immediately after cell membrane disruption. The peptides evaluated

were labelled with fluorescein isothiocyanate and their effect on *E. coli* examined using a confocal laser scanning microscope. The results suggested that the peptide buforin II affected the growth of *E. coli* by penetrating the cell membrane and binding to the DNA and RNA of the target cells. To facilitate membrane binding and disruption, peptides need to have the ability to adopt amphipathic conformation once they interact with bacterial membranes. This is influenced by the composition and arrangement of the acid amino sequence of the peptides (Montesinos & Bardají, 2008). The acid amino sequence of BP100, H-KKLFKKILKYL-NH_2, and the N-terminal derivatisation are thought to increase the antimicrobial activity against the Gram-negative bacteria such as *X. axonopodis* pv. *vesicatoria* (Badosa *et al*., 2007), compared with other peptides.

The composition of the microtitre plates used to assess the effect of peptides on target microorganisms may also affect the efficacy of a peptide. The antimicrobial activity of cationic peptides, such as buforin II, cecropin PI, indolicidin, magainin II, nisin, ranalexin and amikacin against *E. coli*, *Staphylococcuc aureus* and *Pseudomonas aeruginosa* was reported to be higher when treated cells were incubated in polypropylene than in polystyrene tubes (Giacometti *et al*., 2000). In contrast, Sánchez-Gómez *et al*. (2008) found that the composition of the microtitre plates (polypropylene or polystyrene) did not significantly affect the MIC or MBC of peptides P4, P11, P43, P44, P49, P50 and P55 against the growth of Gram-negative pathogens such as *E. coli*, *P. aeruginosa* and *Bordetella bronchiseptica*. In the current work, the composition of microtitre plate used was polypropylene, but its influence on activity of BP100 was not examined and thus might be of interest to include in future research.

The conventional approach to examining the effect of a peptide on bacterial growth, this is the plate-counting technique, is time-consuming, laborious and costly. In the experiments conducted here, the plate-counting technique was found to be useful and reliable when determining the bactericidal effect of the peptide on the target pathogen. Although the turbidimetric technique is easier to use, fast and can be non-destructive, it also involves a high initial cost to purchase the equipment and for maintenance of the equipment. In the current study, the advantages and disadvantages of using these two methods were not examined in detail. A comparison of the two approaches on a range of bacteria would be useful to determine the most suitable method.

In conclusion, peptide BP100 inhibited the growth of DAR75532 in SPB in the range of 2.5 to 10 μM. The bacteriostatic or bactericidal activity of this peptide was affected by the incubation time, the initial inoculum concentration of pathogen cells used and also the concentrations of peptide used.

CHAPTER SIX

General discussion

Of the eight isolates examined in this study, 64161-7, PC397, PC506, PC507, *B. subtilis* and SUPP demonstrated potential to inhibit the growth of DAR75532 *in vitro*. Except for *B. subtilis*, the other bacterial isolates used were obtained from pistachio trees. Further studies of the possible mode of antagonism by the two most promising isolates, 64161L and PC397, suggested that they produced antibacterial metabolites in liquid medium, and that they may have competed with the pathogen. The antibacterial activity of the two isolates studied was affected by the composition of culture medium. When the bacterial isolates were introduced individually to autoclaved excised pistachio twigs by inoculating suspension onto the cut surface, they demonstrated ability to colonise the wood. However, attempts to introduce the pathogen using the same technique failed and led to the development of a vacuum infiltration technique. Inoculum of isolates DAR75532, 64161L and PC397 was successfully introduced into the wood using vacuum infiltration, but the populations appeared to decline after 10 days of incubation. Simultaneous infiltration of the pathogen plus 64161L or PC397 showed that PC397 was more effective than 64161L at reducing colonisation of twigs by the pathogen. In the second part of the current study, peptide BP100 suppressed growth of DAR75532 in liquid medium. The effect was either bacteriostatic or bactericidal and appeared to vary with concentration of pathogen inoculum and the peptide concentration.

Carbon sources were observed to influence the antibiotic activity of the two most promising antagonists in liquid medium. As the use of single sugars and starch as carbon sources in the current experiments may not represent the natural sources of carbon in pistachio trees, it would be appropriate to repeat the experiment using carbohydrates naturally present in pistachio wood. However, there is little information in the literature regarding the availability of carbohydrates in pistachio trees. Most such studies, to date, have focused on the uptake of macronutrients into the tree (Brown *et al.*, 1995; Rosecrance *et al.*, 1996). The survival of the antagonists in the tree might not be of great concern as they were originally isolated from pistachio, but nutrient factors may affect their ability to produce antibiotics or otherwise antagonise DAR75532 and, subsequently, to reduce the population of the pathogen in the tree. Being an alternate bearing plant, the amount of macronutrient and storage and mobilisation of

carbohydrate (composition not defined) in the tree varies during the „on" and „off" cycles (Spann *et al*., 2008b). In a heavy cropping (on-crop) year, accumulation of starch in the branches of mature pistachio trees is reduced by 90% (Rosecrance *et al*., 1998) and most carbohydrate in the branches is mobilised for nut development (Spann *et al*., 2008b). As the nutrients are stored during the „off" year, it might be better to apply an antagonist during this period to facilitate colonisation and survival in the tree.

The simultaneous application of pathogen and the antagonist 64161L or PC397 demonstrated the ability of the antagonist to reduce the colonisation of wood by DAR75532. However, preliminary results indicate that the efficacy of these antagonists improved if inoculated 6 days before the pathogen, suggesting that the antagonists were more effective at preventing colonisation than eradicating the pathogen from wood. Various strains of pathogen may produce different response to biological control agents (Jensen *et al*., 2007). As such, it is important to establish the efficacy of the antagonists against other strains of *Xtp* and evaluation should also be conducted in living trees. Once these factors are assessed, then other aspects of biological control, such as the combination effect and formulation required, might be investigated.

As the antagonists survived in pistachio wood following application to the wound surface, they could be applied during the routine pruning programme of pistachio orchards. However, once applied onto the wound, the antagonist(s) would be exposed to UV light and to changes in the surrounding orchard environment, such as fluctuations in relative humidity, and thus the population might decline before cells can penetrate the thin layer of the wounded surface (O'Brien & Lindow, 1989). Therefore, formulation of inoculum of these bacterial antagonists should be considered prior to their application to wounds on tree. Application of inoculum of *P. fluorescens* that had been freeze-dried and resuspended in water allowed better establishment of populations in pear and apple blossoms than did suspensions of cells prepared from cultures growing actively on artificial media (Stockwell *et al*., 1998). Because bacteria cells from freeze-dried inoculum were prepared through lyophilisation process and exposed to more stress, they may have been more tolerant of dry conditions than the fresh cells (Potts, 1994). Prolonged conducive conditions, such as high humidity, may improve the establishment of bacteria, inoculated as fresh cells, in plants. However, such conditions occur infrequently in the regions of Australia where pistachio is cultivated commercially.

The bacterial antagonists in the current study were evaluated as single isolates on artificial medium and in wood. When evaluated in combination, these isolates might produce different efficacy towards inhibiting *Xtp*. The isolates also may be incompatible with one another. Therefore, the possible complementary or other effects of combining the antagonists should be considered. A mixture of biological control agents which antagonise the pathogen via multiple mechanisms may be more useful and effective than single isolates in suppression of disease. For example, the efficacy of LS213 (a product containing *B. subtilis, B. amyloliquefaciens* and chitosan) to suppress Fusarium wilt in tomato planted in the greenhouse was enhanced by combining the product with an isolate of *P. fluorescens* (Domenech *et al*., 2006). The different mode of antagonism, through production of siderophores, exhibited by *P. fluorescens* improved the beneficial effect of the product. Similar benefit might also occur if antagonists in the present study were shown to be compatible when used in combination.

In view of the increasing interest and research about peptides in plant protection, the use of a peptide as an alternative to the microbial antagonists for control of *Xtp* was examined. The potential of peptide BP100 to inhibit the growth of DAR75532 was demonstrated *in vitro*. The minimum inhibitory concentration for peptide BP100 against *Xtp* and its effect on various isolates of pathogen remain to be determined. To further improve our understanding of this peptide, its ability to prevent infection or eradicate the pathogen from infected trees should also be investigated. BP100 and five other peptides were tested for their ability to reduce infection of detached flowers of pear and apple by the fire blight pathogen *Erwinia amylovora* (Badosa *et al*., 2007). One hour prior to treatment of the flowers with *E. amylovora*, peptides (10 µl of 100 or 200 µM) were applied separately. After 5 days of incubation at high relative humidity, four of the peptides were found to reduce the severity of disease, and BP100 was the most effective, reducing disease severity on apple flowers by 63% and on pear flowers by 74%. The use of BP100 in field trials has not yet been reported. To evaluate the efficacy of BP100 in reducing infection of pistachio by *Xtp*, an assay involving infiltrating the peptide into excised pistachio twigs, similar to that used in this study to assess the bacterial antagonists, could be conducted. In addition, possible phytotoxic effects of BP100 on pistachio must also be assessed. Although the effect of BP100 on plant cells has not been investigated, the peptide at 50 µM caused haemolysis of 3% of human red blood cells, and 22% when 150 µM was tested, showing a low toxicity to animals (Badosa *et al*., 2007).

In addition to research on microbial antagonist and peptides, further studies of the ability of the pathogen to infect and express symptoms on trees should be conducted. As inoculum of DAR75532 below than 10^2 CFU/ml did not multiply over 48 hours of incubation in liquid medium, then the question of the minimum amount of inoculum needed for the pathogen to initiate infection in trees must be addressed. The interaction between amount of pathogen inoculum and infection of the host is acknowledged to be difficult to study as the host variety or cultivar, various aspects of plant health and growth conditions affect the progress of disease in the plant (Birch, 2001). However, this interaction has been indirectly investigated by Sedgley *et al*. (2006). While examining the efficacy of quaternary ammonium bactericide on *Xtp*, the authors observed that, in control treatments, inoculum of 10 µl of *Xtp* at 10^4 to 10^5 CFU/ml failed to initiate infection consistently on the treated trees. Subsequently, *Xtp* at low concentration of 10 to 10^3 cells/ml was detected by quantitative PCR in wood samples obtained from asymptomatic pistachio trees (Scott *et al*., 2009). As *Xtp* was not isolated from some samples shown to be weakly positive by PCR, this suggested that the pathogen could be present at low levels, multiply and express disease symptoms when the conditions are conducive. Bacteria cells communicate with each other through quorum sensing, and the cells activate specific gene expression in response to changes in the environment (Swift *et al*., 1996). The most common protein inducer produced by the Gram-negative bacteria, *N*-acyl-homoserine lactones (AHL), increases as the bacterial population increases, and this will activate certain protein through quorum sensing, leading to the expression of disease (He & Zhang, 2008). Furthermore, the minimal infective dose, as discussed above, might vary with age of the inoculum and with different isolates of *Xtp*.

Once an effective agent(s), either bacteria or peptide, has been identified *in vitro*, the next step would be to test their efficacy on living pistachio trees in the shade-house or greenhouse and, ultimately, in the field. The most common problem reported in the development of biological control agent(s) is the inconsistency of disease suppression in field trials. Many factors can influence the efficacy of biological control agents in field, such as climate condition and plant species or cultivar (Jacobsen *et al*., 2004). In the case of pistachio, it also involves the physiology of the tree associated with the alternate bearing. Encouraging and consistent results in field trials would be followed by the development of an appropriate formulation of the biological control agent. The final stage of this process would be the registration of the biological control agents with the Australian Pesticides and Veterinary Medicines Authority. Registration

requires information about the specificity and ability of the microorganism to reproduce in the environment (Anonymous, 2005). Since peptide is not a living microorganism and has high specificity for bacteria or eukaryotic cells (Glukhov *et al*., 2005), it might be easier to register for commercial use against bacterial plant pathogens than the bacterial antagonists.

In conclusion, information about the potential of bacteria and peptide BP100 was generated during the study. The further research suggested above may provide the extra knowledge required to integrate this approach into a management strategy for pistachio dieback.

APPENDIX 1

Culture media used in the experiments

Sucrose Peptone Agar (Moffett & Croft, 1983)

Sucrose	20 g
Bacteriological peptone	5 g
Potassium phosphate	0.5 g
Magnesium sulphate	0.25 g
Distilled water	1 litre
Agar (Oxoid Technical Agar No 3)	14 g

pH was adjusted to 7.4 with pH meter (Oakton®, Extech Equipment Pty. Ltd., Australia) before autoclaving

Nutrient Agar + Antibiotics (Vu Thanh, pers. comm., 2009)

Nutrient broth (Oxoid)	5.2 g
Agar (Oxoid Technical Agar No 3)	5.6 g
Distilled water	400 ml

The following antibiotics were added once the medium had cooled to 50^{o}C after autoclaving:

1 ml of Cephalexin

0.5 ml of Ampicillin

0.28 ml of Gentomycin

Preparation of antibiotic stock solutions

Cephalexin (10 mg/L): Dissolve 0.25 g Cephalexin Hydrate (Sigma-Aldrich) in 25 ml of 75% ethanol.

Ampicillin (1 mg/L): Dissolve 0.02 g of Ampicillin (Sigma-Aldrich) in 10 ml sterile distilled water

Gentomicin (1.4 mg/L): Dissolve 0.05 g Gentomycin in 10 ml sterile distilled water

REFERENCES

Agrios G. (2005) *Plant Pathology* (Fifth Edition). Elsevier Academic Press, London, UK.

Alabouvette C., Olivain C. and Steinberg C. (2006) Biological control of plant diseases: the European situation. *European Journal of Plant Pathology* **114**: 329-341.

Albareda M., Dardanelli M.S., Sousa C., Megías M., Temprano F. and Rodríguez-Navarro D.N. (2006) Factors affecting the attachment of rhizospheric bacteria to bean and soybean roots. *FEMS Microbiology Letter* **259**: 67-73.

Alfano G., Lewis Ivey M.L., Cakir C., Bos J.I.B., Miller S.A., Madden L.V., Kamoun S. and Hoitink H.A.J. (2007) Systemic modulation of gene expression in tomato by *Trichoderma hamatum* 382. *Phytopathology* **97**: 429-437.

Andrä J., Gutsmann T., Garidel P. and Brandenburg K. (2006) Mechanisms of endotoxin neutralization by synthetic cationic compounds. *Journal of Endotoxin Research* **12**: 261-277.

Andreote F.D., Azevedo J.L. and Araújo W.L. (2009) Assessing the diversity of bacterial communities associated with plants. *Brazilian Journal of Microbiology* **40**: 417-432.

Anonymous (2005) Guidelines for registration of biological agricultural product. http://www.apvma.gov.au (Accessed on January 2010).

Anthony T., Rajesh T., Kayalvizhi N. and Gunasekaran P. (2009) Influence of medium components and fermentation conditions on the production of bacteriocin(s) by *Bacillus licheniformis* AnBa9. *Bioresource Technology* **100**: 872-877.

Araújo W.L., Marcon J., Maccheroni W., van Elsan J.D., van Vuurde J.W.L. and Azevedo J.L. (2002) Diversity of endophytic bacterial populations and their interaction with *Xylella fastidiosa* in citrus plants. *Applied and Environmental Microbiology* **68**: 4906-4914.

Asaka O. and Shoda M. (1996) Biocontrol of *Rhizoctonia solani* damping-off of tomato with *Bacillus subtilis* RB14. *Applied and Environmental Microbiology* **62**: 4081-4085.

Askew D.J. and Laing M.D. (1994) The *in vitro* screening of 118 *Trichoderma* isolates for antagonism to *Rhizoctonia solani* and an evaluation of different environmental sites of *Trichoderma* as sources of aggressive strains. *Plant and Soil* **159**: 277-281.

Badosa E., Ferre R., Planas M., Feliu L., Besalŭ E., Cabrefiga J., Bardají E. and Montesinos E. (2007) A library of linear undecapeptides with bactericidal activity against phytopathogenic bacteria. *Peptides* **28**: 2276-2285.

Badosa E., Ferré R., Francés J., Bardají E., Feliu L., Planas M. and Montesinos E. (2009) Sporicidal activity of synthetic antifungal undecapeptides and control of *Penicillium* rot of apples. *Applied and Environmental Microbiology* **75**: 5563-5569.

Bais H.P, Fall R. and Vivanco J.M. (2004) Biocontrol of *Bacillus subtilis* against infection of Arabidopsis roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin production. *Plant Physiology* **134**: 307-319.

Baker, R. (1968) Mechanisms of biological control of soil-borne pathogens. *Annual Review of Phytopathology* **6**: 263-294.

Barer M.R., Burdess D. and Freeman R. (1992) A study into the mechanism of the Crystal Violet reaction in *Staphylococcus aureus*. *Epidemiology and Infection* **109**: 87-96.

Basha S. and Ulaganathan K. (2002) Antagonism of *Bacillus* species (strain BC121) towards *Curvularia lunata. Current Science* **82**: 1457-1463.

Bell C.R., Dickie G.A. and Chan J.W.Y.F. (1995) Variable response of bacteria isolated from grapevine xylem to control grape crown gall disease *in planta*. *American Journal of Enology and Viticulture* **46**: 499-508.

Berg G., Fritze A., Roskot N. and Smalla K. (2001) Evaluation of potential biocontrol rhizobacteria, from different host plants of *Verticillium dahliae* Kleb. *Journal of Applied Microbiology* **91**: 963-971.

Birch R.G. (2001) *Xanthomonas albilineans* and the antipathogenesis approach to disease control. *Molecular Plant Pathology* **2**: 1-11.

Bizani D. and Brandelli A. (2004) Influence of media and temperature on bacteriocin production by *Bacillus cereus* 8A during batch cultivation. *Applied Microbiology and Biotechnology* **65**: 158-162.

Bonaterra A., Mari M., Casalini L. and Montesinos E. (2003) Biological control of *Monilinia laxa* and *Rhizopus stolonifer* in postharvest of stone fruit by *Pantoea agglomerans* EPS125 and putative mechanisms of antagonism. *International Journal of Food Microbiology* **84**: 93-104.

Bonev B., Hooper J. and Parisot J. (2008) Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. *Journal of Antimicrobial Chemotherapy* **61**:1295-1301.

Borowicz J.J. and Omer Z.S. (2000) Influence of rhizobacterial culture media on plant growth and on inhibition of fungal pathogens. *BioControl* **45**: 355-371.

Brannen P.M. and Kenney D.S. (1997) Kodiak® - a successful biological-control product for suppression of soil-borne plant pathogens of cotton. *Journal of Industrial Microbiology & Biotechnology* **19**: 169-171.

Bressan W. and Figueiredo J.E.F. (2008) Efficacy and dose-response relationship in biocontrol of *Fusarium* disease in maize by *Streptomyces* spp.. *European Journal of Plant Pathology* **120**: 311-316.

Broggini G.A.L., Duffy B., Holliger E., Schärer H.-J., Gessler C. and Patocchi A. (2005) Detection of the fire blight biocontrol agent *Bacillus subtilis* BD170 (Biopro®) in a Swiss apple orchard. *European Journal of Plant Pathology* **111**: 93-100.

Brown, P.H., Weinbaum S.A. and Picchioni G.A. (1995) Alternate bearing influences annual nutrient consumption and the total nutrient content of mature pistachio trees. *Trees-Structure and Function* **9**: 158-164.

Burke, V. (1921) Notes on the gram stain with description of a new method. *Journal of Bacteriology* **7**: 159-182.

Burr T.J., Schroth M.N. and Suslow T. (1978) Increased potato yields by treatment of seedpieces with specific strains of *Pseudomonas fluorescens* and *P. putida*. *Phytopathology* **68**: 1377-1383.

Chapuis L., Richard L. and Dubos B. (1998) Variation in susceptibility of grapevine pruning wound to infection by *Eutypa lata* in south-western France. *Plant Pathology* **47**: 463-472.

Charoensopharat K., Thummabenjapone P., Sirithorn P. and Thammsirirak S. (2008) Antibacterial substance produced by *Streptomyces* sp. No. 87. *African Journal of Biotechnology* **7**: 1362-1368.

Chen C., Bauske E.M., Musson G., Rodríguez-Kábana R. and Kloepper J.W. (1995) Biological control of *Fusarium* wilt on cotton by use of endophytic bacteria. *Biological Control* **5**: 83-91.

Crawford D.L., Lynch J.M., Whipps J.M. and Ousley M.A. (1993) Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Applied and Environmental Microbiology* **59**: 3899-3905.

Cronin D., Moeënne-Loccoz Y., Fenton A., Dunne C., Dowling D.N., and O'Gara F. (1997) Ecological interaction of a biocontrol *Pseudomonas fluorescens* strain producing 2,4-diacetylphloroglucinol with the soft rot potato pathogen *Erwinia carotovora* subsp. *atroseptica*. *FEMS Microbiology Ecology* **23**: 95-106.

Davis M.J., Thomson S.V. and Purcell A.H. (1980) Etiological role of the xylem-limited bacterium causing Pierce‟s disease in almond leaf scorch. *Phytopathology* **70**: 472-475.

De La Fuente L., Thomashow L., Weller D., Bajsa N. and Quagliotto L. (2004) *Pseudomonas fluorescens* UP61 isolated from birdfoot trefoil rhizosphere produces multiple antibiotics and exerts a broad spectrum of biocontrol activity. *European Journal of Plant Pathology* **110**: 671-681.

Domenech J., Reddy M.S., Kloepper J.W., Ramos B. and Gutierrez-Mañero J. (2006) Combined application of the biological product LS213 with *Bacillus*, *Pseudomonas* or *Chryseobacterium* for growth promotion and biological control of soil-borne diseases in pepper and tomato. *BioControl* **51**: 245-258.

Edwards M. and Taylor C. (1998) Dieback and canker in Australian pistachios. *Acta Horticulturae* **470**: 596-603.

El-Hassan S.A. and Gowen S.R. (2006) Formulation and delivery of the bacterial antagonist *Bacillus subtilis* for management of lentil vascular wilt caused by *Fusarium oxysporum* f. sp. *lentis*. *Phytopathology* **154**: 148-155.

Elliott M.L. and Des Jardin E.A. (1999) Comparison of media and diluents for enumeration of aerobic bacteria from Bermuda grass golf course putting greens. *Journal of Microbiological Methods* **34**: 193-202.

Emmert E.A.B. and Handelsman J. (1999) Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters* **171**: 1-9.

Epstein L., Beede R., Kaur S. and Ferguson L. (2003) Rootstock effects on pistachio trees grown in *Verticillium dahliae*-infested soil. *Phytopathology* **94**: 388-395.

Errakhi R., Bouteau F., Lebrihi A. and Barakate M. (2007) Evidences of biological control capacities of *Streptomyces* spp. against *Sclerotium rolfsii* responsible for

damping-off disease in sugar beet (*Beta vulgaris* L.). *World Journal of Microbiology and Biotechnology* **23**: 1503-1509.

Etebarian H.R., Scott E.S. and Wicks T.J. (2000) *Trichoderma harzianum* T39 and *T. virens* DAR 74290 as potential biological control agents for *Phytophthora erythroseptica*. *European Journal of Plant Pathology* **106**: 329-337.

Facelli E., Taylor C., Scott E., Emmett B., Fegan M. and Sedgley M. (2002) Bacterial dieback of pistachio in Australia. *Australasian Plant Pathology* **31**: 95-96.

Facelli E., Taylor C., Scott E., Fegan M., Huys G., Noble R.D., Swings J. and Sedgley M. (2005) Identification of the causal agent of pistachio dieback in Australia. *European Journal of Plant Pathology* **112**: 155-165.

Facelli E., Taylor C., Williams N.M., Emmett R.W., Sedgley M., Joyce C.K. and Scott E.S. (2009) Location of *Xanthomonas translucens* in pistachio trees. *Australasian Plant Pathology* **38**: 584-593.

Ferre R., Badosa E., Feliu L., Planas M., Montesinos E. and Bardají E. (2006) Inhibition of plant-pathogenic bacteria by short synthetic cecropin A-melittin hybrid peptides. *Applied and Environmental Microbiology* **72**: 3302-3308.

Ferreira J.H.S., Matthee F.N. and Thomas A.C. (1991) Biological control of *Eutypa lata* on grapevine by an antagonistic strain of *Bacillus subtilis*. *Phytopathology* **81**: 283-287.

Ferreira R.B., Monteiro S., Freitas R., Santos C.N., Chen Z., Batista L.M., Duarte J., Borges A. and Teixeira A.R. (2006) Fungal pathogens: the battle for plant infection. *Critical Reviews in Plant Science* **25**: 505-524.

Fravel D.R. (1988) Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology* **26**: 75-91.

Fritschi F.B., Lin H. and Walker M.A. (2007) *Xylella fastidiosa* population dynamics in grapevine genotypes differing in susceptibility to Pierce‟s disease. *American Journal of Enology and Viticulture* **58**: 326-332.

Gamard P. and De Boer S.H. (1995) Evaluation of antagonistic bacteria for suppression of bacterial ring rot of potato. *European Journal of Plant Pathology* **101**: 519-525.

Gardner J.M., Feldman A.W. and Zablotowicz R.M. (1982) Identity and behavior of xylem-residing bacteria in rough lemon roots of Florida citrus trees. *Applied and Environmental Microbiology* **43**: 1335-1342.

Giacometti A., Cirioni O., Barchiesi F., del Prete M.S., Fortuna M., Caselli F. and Scalise G. (2000) *In vitro* susceptibility tests for cationic peptides: comparison of broth microdilution methods for bacteria that grow aerobically. *Antimicrobial Agents and Chemotherapy* **44**: 1694-1696.

Giblot-Ducray D., Marefat A., Gillings M.R., Parkinson N.M., Bowman J.P., Ophel-Keller K., Taylor C., Facelli E. and Scott E.S. (2009) Proposal of *Xanthomonas translucens* pv. *pistaciae* pv. *nov*., pathogenic to pistachio (*Pistacia vera*). *Systematic and Applied Microbiology* **32**: 549-557.

Gilbert A. (2005) *Just nuts*. Hyland House Publishing Pty Ltd, Victoria.

Giotis C., Markelou E., Theodoropoulou A., Toufexi E., Hodson R., Shotton P., Shiel R., Cooper J. and Leifert C. (2009) Effect of soil amendments and biological control agents (BCAs) on soil-borne root disease caused by *Pyrenochaeta lycopersici* and *Verticillium albo-atrum* in organic greenhouse tomato production systems. *European Journal of Plant Pathology* **123**: 387-400.

Glukhov E., Stark M., Burrows L.L. and Deber C.M. (2005) Basis for selectivity of cationic antimicrobial peptides for bacterial versus mammalian membranes. *Journal of Biological Chemistry* **280**: 33960-33967.

Gregersen T. (1978) Rapid method for distinction of Gram-negative from Gram-positive bacteria. *European Journal of Applied Microbiology and Biotechnology* 5: 123-127.

Greiner M. and Winkelmann G. (1991) Fermentation and isolation of herbicolin A, a peptide antibiotic produced by *Erwinia herbicola* strain A111. *Applied Microbiology and Biotechnology* **34**: 565-569.

Gurusiddaiah S., Weller D.M., Sarkar A. and Cook R.J. (1986) Characterization of an antibiotic produced by a strain of *Pseudomonas fluorescens* inhibitory to *Gaeumannomyces graminis* var. *tritici* and *Pythium* spp.. *Antimicrobial Agents and Chemotherapy* **29**: 488-495.

Haapalainen M., Mattinen J. and Metzler M.C. (2000) The growth of a plant-parasitic bacterium, *Clavibacter xyli* subsp. c*ynodontis*, is enhanced by xylem fluid components. *Physiological and Molecular Plant Pathology* **56**: 147-155.

Hammami I., Rhouma A., Jaouadi B., Rebai A. and Nesme X. (2009) Optimization and biochemical characterization of a bacteriocin from a newly isolated *Bacillus subtilis* strain 14B for biocontrol of *Agrobacterium* spp. strains. *Letters in Applied Microbiology* **48**: 253-260.

Hartzen S.H., Andersen L.P., Bremmelgaard A., Colding H.M., Arpi M., Kristiansen J., Justesen T., Espersen F., Frimodt-Møller N. and Bonnevie O. (1997) Antimicrobial susceptibility testing of 230 *Helicobacter pylori* strains: importance of medium, inoculum, and incubation time. *Antimicrobial Agents and Chemotherapy* **41**: 2634–2639.

He Y.W. and Zhang L.H. (2008) Quorum sensing and virulence in *Xanthomonas campestris*. *FEMS Microbiology Review* **32**: 842-857.

Hélias V., Andrivon D. and Jouan B. (2000) Development of symptoms caused by *Erwinia carotovora* ssp. *atroseptica* under field conditions and their effects on the yield of individual potato plants. *Plant Pathology* **49**: 23-32.

Hendricks L. and Ferguson L. (1995) *The pistachio tree, Pistachio production*. Centre for Fruit and Nut Crop Research Information, University of California Davis, USA.

Holtsmark I., Eijsink V.G.H. and Bente Brurberg M. (2008) Bacteriocins from plant pathogenic bacteria. *FEMS Microbiology Letters* **280**: 1-7.

Homma Y., Sato Z., Hirayama F., Konno K., Shirahama H. and Suzui T. (1989) Production of antibiotics by *Pseudomonas cepacia* as an agent for biological control of soilborne plant pathogens. *Soil Biology and Biochemistry* **21**:723-728.

Howell C.R. and Stipanovic R.D. (1979) Control of *Rhizoctonia solani* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. *Phytopathology* **69**: 480-482.

Ishimaru C.A., Klos E.J. and Brubaker R.R. (1988) Multiple antibiotic production by *Erwinia herbicola. Phytopathology* **78**: 746-750.

Israel S., Mawar R. and Lodha S. (2005) Soil solarisation, amendments and bio-control agents for the control of *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *cumini* in arid soils. *Annals of Applied Biology* **146**: 481-491.

Jacobsen B.J., Zidack N.K. and Larson B.J. (2004) The role of *Bacillus*-based biological control agents in integrated pest management systems: plant diseases. *Phytopathology* **94**: 1272-1275.

Jensen D.F., Knudsen I.M.B., Lübeck M., Mamarabadi M., Hockenhull J. and Jensen B. (2007) Development of a biocontrol agent for plant disease control with special emphasis on the near commercial fungal antagonist *Clonostachys rosea* strain IK726. *Australasian Plant Pathology* **36**: 95-101.

Jock S., Völksch B., Mansvelt L. and Geider K. (2002) Characterization of *Bacillus* strains from apple and pear trees in South Africa antagonistic to *Erwinia amylovora. FEMS Microbiology Letters* **211**: 247-252.

John S., Scott E.S., Wicks T.J. and Hunt J.S. (2004) Interactions between *Eutypa lata* and *Trichoderma harzianum*. *Phytopathologia Mediterranea* **43**: 95-104.

John S., Wicks T.J., Hunt J.S., Lorimer M.F., Oakey H. and Scott E.S. (2005) Protection of grapevine pruning wounds from infection by *Eutypa lata* using *Trichoderma harzianum* and *Fusarium lateritium. Australasian Plant Pathology* **34**: 569-575.

Johnson K.B. and Stockwell V.O. (1998) Management of fire blight: a case study in microbial ecology. *Annual Review of Phytopathology* **36**: 227-248.

Kaprelyants A.S. and Kell D.B. (1996) Do bacteria need to communicate with each other for growth? *Trends in Microbiology* **4**: 237-242.

Kearns L.P. and Mahanty H.K. (1998) Antibiotic production by *Erwinia herbicola* Eh1087: its role in inhibition of *Erwinia amylovora* and partial characterization of antibiotic biosynthesis genes. *Applied and Environmental Microbiology* **64**: 1837-1844.

Klaenhammer T.R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiology Reviews* **12**: 39-86.

Kobayashi D.Y., Guglielmoni M. and Clarke B.B. (1995) Isolation of the chitinolytic bacteria *Xanthomonas maltophilia* and *Serratia marcescens* as biological control agents for summer patch disease of turfgrass. *Soil Biology and Biochemistry* **27**: 1479-1487.

Korsten L., De Villiers E.E., Wehner F.C. and Kotzé J.M. (1997) Field sprays of *Bacillus subtilis* and fungicides for control of preharvest fruit diseases of avocado in South Africa. *Plant Disease* **81**: 455-459.

Lee K.J., Kamala-Kannan S., Sub H.S., Seong C.K. and Lee G.W. (2008) Biological control of *Phytophthora* blight in red pepper (*Capsicum annuum* L.) using

Bacillus subtilis. *World Journal of Microbiology and Biotechnology* **24**: 1139-1145.

Leelasuphakul W., Sivanunsakul P. and Phongpaichit S. (2006) Purification, characterization and synergistic activity of β-1,3-glucanase and antibiotic extract from an antagonistic *Bacillus subtilis* NSRS 89-24 against rice blast and sheath blight. *Enzyme and Microbial Technology* **38**: 990-997.

Leelasuphakul W., Hemmanee P. and Chuenchitt S. (2008) Growth inhibitory properties of *Bacillus subtilis* strains and their metabolites against the green mold pathogen (*Penicillium digitatum* Sacc.) of citrus fruit. *Postharvest Biology and Technology* **48**: 113-121.

Lehtinen J., Järvinen S., Virta M. and Lilius E.M. (2006) Real-time monitoring of an antimicrobial activity with the multiparameter microplate assay. *Journal of Microbiological Methods* **66**: 381-389.

Lemanceau P., Bauer P., Kraemer S. and Briat J.F. (2009) Iron dynamics in the rhizosphere as a case study for analysing interactions between soils, plants and microbes. *Plant and Soil* **321**: 513-535.

Li J.Y., Wang J.H. and Wang H.M. (2009) Mode of action of the antibacterial compound Ar26 produced by *Agrobacterium vitis* strain E26 with activity against *A. tumefaciens*, *A. rhizogenes* and *A. vitis*. *Journal of Phytopathology* **157**: 159-165.

Lin D., Qu L.–J., Gu H. and Chen Z. (2001) A 3.1-kb genomic fragment of *Bacillus subtilis* encodes the protein inhibiting growth of *Xanthomonas oryzae* pv. *oryzae*. *Journal of Applied Microbiology* **91**: 1044-1050.

Lodewyckx C., Vangronsveld J., Porteous F., Moore E.R.B., Taghavi S., Mezgeay M. and van der Lelie D. (2002) Endophytic bacteria and their potential applications. *Critical Reviews in Plant Science* **21**: 583-606.

Loper J.E., Kobayashi D.Y. and Paulsen I.T. (2007) The genomic sequence of *Pseudomonas fluorescens* Pf-5: insights into biological control. *Phytopathology* **97**: 233-238.

Maccagnani B., Giacomello F., Fanti M., Gobbin D., Maini S. and Angeli G. (2009) *Apis mellifera* and *Osmia cornuta* as carriers for the secondary spread of *Bacillus subtilis* on apple flowers. *BioControl* **54**: 123-133.

Marchi G., Sisto A., Cimmino A., Andolfi A., Cipriani M.G., Evidente A. and Surico G. (2006) Interaction between *Pseudomonas savastanoi* pv. *savastanoi* and *Pantoea agglomerans* in olive knots. *Plant Pathology* **55**: 614-624.

Marcos J.F., Muñoz A., Pérez-Payá E., Misra S. and López-García B. (2008) Identification and rational design of novel antimicrobial peptides for plant protection. *Annual Review of Phytopathology* **46**: 273-301.

Marefat A., Ophel-Keller K., Scott E.S. and Sedgley M. (2006a) The use of ARMS PCR in detection and identification of xanthomonads associated with pistachio dieback in Australia. *European Journal of Plant Pathology* **116**: 57-68.

Marefat A., Scott E.S., Ophel-Keller K. and Sedgley M. (2006b) Genetic, phenotypic and pathogenic diversity among xanthomonads isolated from pistachio (*Pistachia vera*) in Australia. *Plant Pathology* **55**: 639-649.

Mari M., Guizzardi M. and Pratella G.C. (1996) Biological control of gray mold in pears by antagonistic bacteria. *Biological Control* **7**: 30-37.

Marten P., Smalla K. and Berg G. (2000) Genotype and phenotype differentiation of an antifungal biocontrol strain belonging to *Bacillus subtilis. Journal of Applied Microbiology* **89**: 463-471.

McManus P., Stockwell V.O., Sundin G.W. and Jones A.L. (2002) Antibiotic use in plant agriculture. *Annual Review of Phytopathology* **40**: 443-465.

McSpadden Gardener B.B. (2007) Diversity and ecology of biocontrol *Pseudomonas* spp. in agricultural systems. *Phytopathology* **97**: 221-226.

Mellouli L., Karray-Rebai I., Sioud S., Ameur-Mehdi R., Naili B. and Bejar S. (2004) Efficient transformation procedure of a newly isolated *Streptomyces* sp. TN58 strain producing antibacterial activities. *Current Microbiology* **49**: 400-406.

Mercado-Blanco J. and Bakker P.A.H.M. (2007) Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek* **92**: 367-389.

Mila A.L. and Michailides T.J. (2006) Use of Bayesian methods to improve prediction of panicle and shoot blight severity of pistachio in California. *Phytopathology* **96**: 1141-1147.

Moffett M. and Croft B. (1983) *Xanthomonas* pp. 189-228, In: Fahy P. and Persley G (eds) *Plant Bacterial Diseases. A Diagnostic Guide*. Academic Press, Sydney.

Monroc S., Badosa E., Feliu L., Planas M., Montesinos E. and Bardají E. (2006) *De novo* designed cyclic cationic peptides as inhibitors of plant pathogenic bacteria. *Peptides* **27**: 2567-2574.

Montesinos E. (2007) Antimicrobial peptides and plant disease control. *FEMS Microbiology Letters* **270**: 1-11.

Montesinos E. and Bardají E. (2008) Synthetic antimicrobial peptides as agricultural pesticides for plant disease control. *Chemistry and Biodiversity* **5**: 1225-1237.

Moss W.P., Byrne J.M., Campbell H.L., Ji P., Bonas U., Jones J.B. and Wilson M. (2007) Biological control of bacterial spot of tomato using *hrp* mutants of *Xanthomonas campestris* pv. *vesicatoria*. *Biological Control* **41**: 199-206.

Munkvold G.P. and Marois J.J. (1993) Efficacy of natural epiphytes and colonizers of grapevine pruning wounds for biological control of eutypa dieback. *Phytopathology* **83**: 624-629.

Muñoz A., López-García B. and Marcos J.F. (2007) Comparative study of antimicrobial peptides to control citrus postharvest decay caused by *Penicillium digitatum*. *Journal of Agriculture and Food Chemistry* **55**: 8170-8176.

Nagórska K., Bikowski M. and Obuchowski M. (2007) Multicellular behaviour and production of a wide variety of toxic substances support usage of *Bacillus subtilis* as a powerful biocontrol agent. *Acta Biochimica Polonica* **54**: 495-508.

Neeno-Eckwall E., Kinkel L.L. and Schottel J.L. (2001) Competition and antibiosis in the biological control of potato scab. *Canadian Journal of Microbiology* **47**: 332-340.

Oard S.V. and Enright F.M. (2006) Expression of the antimicrobial peptides in plants to control phytopathogenic bacteria and fungi. *Plant Cell Reports* **25**: 561-572.

O'Brien R.D. and Lindow S.E. (1989) Effect of plant species and environmental conditions on epiphytic population sizes of *Pseudomonas syringae* and other bacteria. *Phytopathology* **79**: 619-627.

Okigbo R.N. and Ikediugwu F.E.O. (2000) Studies on biological control of postharvest rot in yams (*Dioscorea* spp.) using *Trichoderma viride*. *Journal of Phytopathology* **148**: 351-355.

Parente E., Brienza C., Moles M. and Ricciardi A. (1995) A comparison of methods for the measurement of bacteriocin activity. *Journal of Microbiological Methods* **22**: 95-108.

Park C. B., Kim H.S. and Kim S. C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. *Biochemical and Biophysical Research Communications* **244:** 253–257.

Paulitz T.C. and Bélanger R.R. (2001) Biological control in the greenhouse system. *Annual Review of Phytopathology* **39**: 103-133.

Pijls C.F.N., Shaw M.W. and Parker A. (1994) A rapid test to evaluate *in vitro* sensitivity of *Septoria tritici* to flutriafol, using a microtitre plate reader. *Plant Pathology* **43**: 726-732.

Pistachio Growers Association Incorporation (2008) http://www.pgai.com.au (Accessed on January 2010).

Poppe L., Vanhoutte S. and Höfte M. (2003) Modes of action of *Pantoea agglomerans* CPA-2, an antagonist of postharvest pathogens on fruits. *European Journal of Plant Pathology* **109:** 963–973.

Potts M. (1994) Desiccation tolerance of prokaryotes. *Microbiological Reviews* **58**: 755-805.

Rajasekaran K., Stromberg K.D., Cary J.W. and Cleveland T.E. (2001) Broad-spectrum antimicrobial activity *in vitro* of the synthetic peptide D4E1. *Journal of Agricultural and Food Chemistry* **49**: 2799-2803.

Rajkumar M., Lee W.H. and Lee K.J. (2005) Screening of bacterial antagonists for biological control of *Phytophthora* blight of pepper. *Journal of Basic Microbiology* **45**:55-63.

Ramesh R., Joshi A.A. and Ghanekar M.P. (2009) Pseudomonads: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.). *World Journal of Microbiology and Biotechnology* **25**: 47-55.

Reiter B., Wermbter N., Gyamfi S., Schwab H. and Sessitsch A. (2003) Endophytic *Pseudomonas* spp. populations of pathogen-infected potato plants analysed by 16S rDNA- and 16S rRNA-based denaturating gradient gel electrophoresis. *Plant and Soil* **257**: 397-405.

Rice K. C. and Bayles K.W. (2008) Molecular control of bacterial death and lysis. *Microbiology and Molecular Biology Reviews* **72**: 85-109.

Robinson B. (1998) Pistachios pp. 436-443, In: Hyde K. (ed) The new rural industries: a handbook for farmers and investors. RIRDC, Canberra, Australia.

Rosecrance R.C., Weinbaum S.A. and Brown P.H. (1996) Assessment of nitrogen, phosphorus, and potassium uptake capacity and root growth in mature alternate-bearing pistachio (*Pistacia vera*) trees. *Tree Physiology* **16**: 949-956.

Rosecrance R.C., Weinbaum S.A. and Brown P.H. (1998) Alternate bearing affects nitrogen, phosphorus, potassium and starch storage pools in mature pistachio trees. *Annals of Botany* **82**: 463-470.

Ryan R.B., Germaine K., Franks A., Ryan D.J. and Dowling D.N. (2008) Bacterial endophytes: recent developments and applications. *FEMS Microbiology Letters* **278**: 1-9.

Sánchez-Gómez S., Lamata M., Leiva J., Blondelle S.E., Jerala R., Andrä J., Brandenburg K., Lohner K., Moriyon I. and Martínez-de-Tejada G. (2008) Comparative analysis of selected methods for the assessment of antimicrobial and membrane-permeabilizing activity: a case study for lactoferricin derived peptides. *BMC Microbiology* **8**:196

Scherm H., Savelle A.T. and Law S.E. (2007) Effect of electrostatic spray parameters on the viability of two bacterial biocontrol agents and their deposition on blueberry flower stigmas. *Biocontrol Science and Technology* **17**: 285-293.

Schisler D.A., Slininger P.J., Hanson L.E. and Loria R. (2000) Potato cultivar, pathogen isolates and antagonist cultivation medium influence the efficacy and ranking of bacterial antagonists of *Fusarium* dry rot. *Biocontrol Science and Technology* **10**: 267-279.

Schmidt C.S., Lorenz D. and Wolf G.A. (2001a) Biological control of the grapevine dieback fungus *Eutypa lata* I: screening of bacterial antagonists. *Journal of Phytopathology* **149**: 427-435.

Schmidt C.S., Lorenz D., Wolf G.A. and Jäger J. (2001b) Biological control of the grapevine dieback fungus *Eutypa lata* II: influence of formulation additives and transposon mutagenesis on the antagonistic activity of *Bacillus subtilis* and *Erwinia herbicola*. *Journal of Phytopathology* **149**: 437-445.

Schottel J.L., Shimizu K. and Kinkel L.L. (2001) Relationship of *in vitro* pathogen inhibition and soil colonization to potato scab biocontrol by antagonistic *Streptomyces* spp.. *Biological Control* **20**: 102-112.

Schubert M., Fink S. and Schwarze F.W.M.R. (2008) Evaluation of *Trichoderma* spp. as a biocontrol agent against wood decay fungi in urban trees. *Biological Control* **45**: 111-123.

Scott E., Giblot-Ducray D., Emmett B., Taylor C. and Ophel-Keller K. (2009) Challenges to the Australian pistachio industry – bacterial dieback and nut quality. *Final Report to Horticulture Australia Ltd* (Project PS06002).

Sedgley M., Scott E., Facelli E., Anderson N., Emmett B. and Taylor C. (2006) Pistachio canker epidemiology. *Final Report to Horticulture Australia Ltd* (Project PS04015).

Shoda M. (2000) Bacterial control of plant disease. *Journal of Bioscience and Bioengineering* **89**: 515-521.

Shortle W.C. and Cowling E.B. (1978) Interaction of live sapwood and fungi commonly found in discoloured and decayed wood. *Phytopathology* **68**: 617-623.

Singh G., Maurya S., De Lampasona M.P. and Catalan C. (2005) Chemical constituents, antimicrobial investigations, and antioxidative potentials of *Anethum graveolens* L. essential oil and acetone extract: Part 52. *Journal of Food Science* **70**: 208-215.

Smith C.J. (1996) Accumulation of phytoalexins: defence mechanism and stimulus response system. *New Phytologist* **132**: 1-45.

Spann T.M., Beede R.H. and Dejong T.M. (2008a) Neoformed growth responses to dormant pruning in mature and immature pistachio trees grown on different rootstocks. *Journal of Horticultural Science & Biotechnology* **83**: 137-142.

Spann T.M., Beede R.H. and Dejong T.M. (2008b) Seasonal carbohydrate storage and mobilization in bearing and non-bearing pistachio (*Pistacia vera*) trees. *Tree Physiology* **28**: 207-213.

Stevens D.A. (1984) Antifungal drug susceptibility testing. *Mycopathologia* **87**: 135-140.

Stewart A. (2001) Commercial biocontrol – reality or fantasy? *Australian Plant Pathology* **30**: 127-131.

Stockwell V.O., Johnson K.B. and Loper J.E. (1998) Establishment of bacterial antagonists of *Erwinia amylovora* on pear and apple blossoms as influenced by inoculum preparation. *Phytopathology* **88**: 506-513.

Stockwell V.O. and Stack J.P. (2007) Using *Pseudomonas* spp. for integrated biological control. *Phytopathology* **97**: 244-249.

Swift S., Throup J.P., Williams P., Salmond G.P.C. and Stewart G.S.A.B. (1996) Quorum sensing: a population density component in the determination of bacterial phenotype. *Trends in Biochemical Science* **21**: 214-219

Swinnen I.A.M., Bernaerts K., Dens E.J.J., Geeraerd A.H. and van Impe J.F. (2004) Predictive modelling of the microbial lag phase: a review. *International Journal of Food Microbiology* **94**: 137-159.

Tam V.H., Louie A., Deziel M.R., Liu W., Leary R. and Drusano G.L. (2005) Bacterial-population responses to drug-selective pressure: examination of garenoxacin‟s effect on *Pseudomonas aeruginosa. Journal of Infectious Disease* **192**: 420-428.

Tamehiro N., Okamoto-Hosoya Y., Okamoto S., Ubukata M., Hamada M., Naganawa H. and Ochi K. (2002) Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. *Antimicrobial Agents and Chemotherapy* **46**: 315–320.

Taylor C. and Edwards M. (2000) Managing canker and dieback in pistachios. *Final Report to Horticulture Australia Ltd* (Project NT97002).

Taylor C., Facelli E., Scott E.S., Emmett R.W. and Sedgley M. (2005) Spread and control of pistachio dieback in Australia pp.183-185, In: Oliviera M.M. and CordeiroV. (eds) XIII GREMPA Meeting on Almonds and Pistachios. *Options Méditerranéennes* Number 63.

Taylor C., Giblot Ducray D., Ophel-Keller K., Herdina, Emmet B. and Scott E. (2007) Towards better management and control of pistachio dieback. *Australian Nutgrower* **21**: 24-26.

Temple T.N., Stockwell V.O., Loper J.E. and Johnson K.B. (2004) Bioavailability of iron to *Pseudomonas fluorescens* strain A506 on flowers of pear and apple. *Phytopathology* **94**: 1286-1294.

Teviotdale B.L. (1995) *Soil-borne diseases, Pistachio production*. Centre for Fruit and Nut Crop Research Information, University of California Davis, USA.

Trigiano R.N., Windham M.T. and Windham A.S. (eds) (2004) *Plant pathology: concepts and laboratory exercises.* CRC Press, Tennessee.

Trivedi P., Pandey A. and Palni L.M.S. (2008) *In vitro* evaluation of antagonistic properties of *Pseudomonas corrugata*. *Microbiological Research* **163**: 329-336.

Ubalua A.O. and Oti E. (2007) Antagonistic properties of *Trichoderma viride* on post harvest cassava root rot pathogens. *African Journal of Biotechnology* **6**: 2447-2450.

Uppal A.K., El-Hadrami A., Adam L.R., Tenuta M. and Daayf F. (2008) Biological control of potato *Verticillium* wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. *Biological Control* **44**: 90-100.

Vanneste J.L., Yu J. and Beer S.V. (1992) Role of antibiotic production by *Erwinia herbicola* Eh252 in biological control of *Erwinia amylovora*. *Journal of Bacteriology* **174**: 2785-2796.

Vega F.E., Pava-Ripoll M., Posada F. and Buyer J.S. (2005) Endophytic bacteria in *Coffea arabica* L.. *Journal of Basic Microbiology* **5**: 371-380.

Velmurugan N., Choi M.S., Han S.S. and Lee Y.S. (2009) Evaluation of antagonistic activities of *Bacillus subtilis* and *Bacillus licheniformis* against wood-staining fungi: *in vitro* and *in vivo* experiments. *The Journal of Microbiology* **47**: 385-392.

Velusamy P., Immanuel J.E., Gnanamanickam S.S. and Thomashow L. (2006) Biological control of rice bacterial blight plant-associated bacteria producing 2,4-diacetylphloroglucinol. *Canadian Journal of Microbiology* **52**: 56-65.

Verhagen B.W.M., Trotel-Aziz P., Couderchet M., Höfte M. and Aziz A. (2010) *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is associated with induction and priming of defence. *Journal of Experimental Botany* **61**: 249-260.

Vilches C., Mendez C. and Hardisson Salas J.A. (1990) Biosynthesis of oleandomycin by *Streptomyces antibioticus*: influence of nutritional conditions and development of resistance. *Journal of General Microbiology* **136**:1447-1454.

Walberg M., Gaustad P. and Steen H.B. (1996) Rapid flow cytometric assessment of mecillinam and ampicillin bacterial susceptibility. *Journal of Antimicrobial Chemotherapy* **37**: 1063-1075.

Wei Q., Li J.Y., Wang J.H. and Wang H.M. (2009) Strain E26 of *Agrobacterium vitis*, a biological control agent of grapevine crown gall, does not contain *virA* and *virG* pathogenis determinants. *Journal of Phytopathology* **157**: 657-665.

Weindling R. (1932) *Trichoderma lignorum* as a parasite of other soil fungi. *Phytopathology* **22**: 837-845.

Whipps J.M., Hand P., Pink D. and Bending G.D. (2008) Phyllosphere microbiology with special reference to diversity and plant genotype. *Journal of Applied Microbiology* **105**: 1744-1755.

Whitesides S.K. and Spotts R.A. (1991) Frequency, distribution, and characteristics of endophytic *Pseudomonas syringae* in pear trees. *Phytopathology* **81**: 453-457.

Wilson M. and Lindow S.E. (1993) Interactions between the biological control agent *Pseudomonas fluorescens* A506 and *Erwinia amylovora* in pear blossoms. *Phytopathology* **83**: 117-123.

Wright S.A.I., Zumoff C.H., Schneider L. and Beer S.V. (2001) *Pantoea agglomerans* strain EH318 produces two antibiotics that inhibit *Erwinia amylovora in vitro*. *Applied and Environmental Microbiology* **67**: 284-292.

Wu H., Wang S., Qiao J., Liu J., Zhan J. and Gao X. (2009) Expression of $HpaG_{Xooc}$ protein in *Bacillus subtilis* and its biological functions. *Journal of Microbiology and Biotechnology* **19**: 194-203.

Wulff E.G., Mguni C.M., Mansfeld-Giese K., Fels J., Lübeck M. and Hockenhull J. (2002a) Biochemical and molecular characterization of *Bacillus amyloliquefaciens*, *B. subtilis* and *B. pumilus* isolates with distinct antagonistic potential against *Xanthomonas campestris* pv. *campestris. Plant Pathology* **51**: 574-584.

Wulff E.G., Mguni C.M., Mortensen C.N., Keswani C.L. and Hockenhull J. (2002b) Biological control of black rot (*Xanthomonas campestris* pv. *campestris*) of brassicas with an antagonistic strain of *Bacillus subtilis* in Zimbabwe. *European Journal of Plant Pathology* **108**: 317-325.

Yilmaz M., Soran H. and Beyatli Y. (2006) Antimicrobial activities of some *Bacillus* spp. strains isolated from the soil. *Microbiological Research* **161**: 127-131.

Yu G.Y., Sinclair J.B., Hartman G.L. and Bertagnolli B.L. (2002) Production of iturin A by *Bacillus amyloliquefaciens* suppressing *Rhizoctonia solani. Soil Biology and Biochemistry* **34**: 955-963.

Zhang S., Raza W., Yang X., Hu J., Huang Q., Xu Y., Liu X., Ran W. and Shen Q. (2008a) Control of *Fusarium* wilt disease of cucumber plants with the application of a bioorganic fertilizer. *Biology and Fertility of Soils* **44**: 1073-1080.

Zhang T., Shi Z.Q., Hu L.B., Cheng L.G. and Wang F. (2008b) Antifungal compounds from *Bacillus subtilis* B-FS06 inhibiting the growth of *Aspergillus flavus*. *World Journal of Microbiology and Biotechnology* **24**: 783-788.